miR-122 BINDING OF HEPATITIS C VIRUS 5' UNTRANSLATED REGION AUGMENTS THE HCV LIFE CYCLE INDEPENDENT FROM THE P-BODY PROTEIN DDX6, AND REPRESENTS A NOVEL TARGET FOR siRNA TARGETED THERAPY

A Thesis submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy in the Department of Microbiology and Immunology of the University of Saskatchewan, Saskatoon, Saskatchewan, Canada

By

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ABSTRACT

Generally Hepatitis C Virus tropism is limited to hepatocytes. This limited tropism is a result of the receptors HCV requires for cellular entry and other host cellular factors including, uniquely, a liver specific miRNA, miR-122. The relationship between HCV and miR-122 is interesting, as commonly, miRNA are associated with suppression of function, but in the case of HCV, miR-122 actively promotes HCV proliferation. In-depth studies have demonstrated that miR-122 along with the RNA induced silencing complex (RISC) protein Argonaute 2 (Ago2) binds directly to two seed sequences separated by 8-9 nucleotides on HCV 5'UTR. Binding to the 5'UTR results in an increase in viral replication and translation. The method by which miR-122 promotes HCV translation and replication is not fully understood but evidence suggests that part of the function of miR-122 is to stabilize the HCV genome and protect it from exonuclease degradation by Xrn1, but other mechanisms remain to be identified. The reliance of HCV on miR-122 is best exemplified by the fact that removal of miR-122 by a miR-122 antagonist drastically impedes HCV viral titers in Chimpanzees and humans with no indication of escape mutants.

The observation that HCV augmentation of the HCV life cycle by miR-122 requires Ago2 suggests that other components downstream in the miRNA suppression pathway may also be part of the mechanism of action. Our studies focused specifically on the processing body (p-body) associated DEAD-box helicase DDX6. DDX6 is essential for p-body assembly, required for robust miRNA suppression activity and elevated in HCV associated hepatocellular carcinomas. As such we hypothesized that DDX6 and p-bodies were directly or in-directly associated with the mechanism of action of miR-122.

Knocking down DDX6 with siRNA indicated that DDX6 augments both HCV replication and translation. To examine whether DDX6 augmentation of HCV replication was related to the effects of miR-122 on the HCV life cycle, HCV replication and translation were assessed in the presence or absence of miR-122 when DDX6 was knocked down. Our data indicated that HCV replication and translation were augmented equally by miR-122 whether DDX6 was present or not. Our data also

demonstrated that HCV replication and translation that was occurring independent of miR-122 was also still affected by DDX6 knockdown. Taken together our observations strongly suggest that the role DDX6 has on HCV is independent of HCV and miR-122's relationship.

In order to better understand miR-122's relationship with HCV, we hypothesized that targeting the miR-122 binding region with siRNA would inhibit HCV replication initially, but that over the course of several rounds of treatment with the same siRNA, HCV would mutate to escape the siRNA, producing escape mutants that replicate without a dependency on miR-122. These escape mutants could be evaluated on how they replicate without using miR-122, shedding light on miR-122 and HCV's relationship. Conversely if no escape mutants arose the siRNA could be further studied as a potential therapeutic for HCV.

siRNA designed to target the miR-122 binding region inhibited HCV replication, confirming that the designed siRNAs could access the miR-122 binding region and function as an siRNA. Interestingly, when the siRNAs were used against a replication competent HCV RNA having a single nucleotide mutation in the first miR-122 binding site, instead of abolishing siRNA knockdown, two of the siRNA showed enhanced inhibition activity. The target sequences of these siRNAs spanned both miR-122 binding sites and we speculate that their inhibitory activity was due to competition for miR-122 binding to site 2. This observation indicates that siRNA targeting the miR-122 binding region have dual activity, by siRNA induced cleavage, and as a competitive inhibitor of miR-122 binding.

Selection for viral escape mutants of the miR-122-binding site targeting siRNAs revealed viral RNAs having mutations within the miR-122 binding sites, in the surrounding region, and to other areas within the HCV IRES. The mutant viruses will be used to assess the influence of miR-122 binding site mutations on HCV replicative fitness, and to determine if the virus can evolve to replicate independent from augmentation by miR-122.

ACKNOWLEDGEMENT

First, I would like to thank my mentor and supervisor Dr. Joyce Wilson. Joyce has been the biggest influence in the development of all my scientific abilities. From bench top to thought process, she has fostered a great environment to think freely; express ideas; and, most importantly, allowed me to follow up on them regardless of past outcomes. Joyce afforded me the opportunity to attend many conferences during my graduate studies, which I am truly grateful.

I would also like to thank the following current and past lab members: Chao Zhang, Travis Marfleet, Kathleen Mitchelson, Yuqi Gu, and Patricia Thibault. I would like to especially thank Patricia, she has been forced to proofread my terrible writing throughout her graduate studies. I cannot thank her enough for her skilled editorial work. She has also contributed immensely to my project through productive scientific conversation; covering for me on weekends and holidays; and on occasion performing experiments for me.

I would like to thank my committee members: Dr. Suresh Tikoo, Dr. Sylvia van Drunen Little-van den Hurk, Dr. Vikram Misra and, Dr. Peter Bretscher. My committee has provided me direction and productive feedback throughout my project.

I would like to express my appreciation to everyone at the Vaccine and Infectious Disease Organization (VIDO) and within the Department of Microbiology for organizing meetings and providing administrative assistance whenever I required it. A special thanks is reserved for Laura Latimer, who helped me find everything I needed around VIDO when I first started. I could not have asked for a better "work mom".

I thank all of the graduate students I shared an office with. A graduate student office can sometimes be a place of distraction, but it also provides a setting for constructive conversations with your peers regarding scientific problems. I was lucky to share offices with some great scientists; and more importantly, some wonderful people. I will carry these friendships with me for the rest of my life.

Lastly, I would like to thank my family. My parents, Joanne and Harry, who have always encouraged and supported my educational dreams. Jennifer, the woman

I married halfway through this project, has been there for me everyday of my graduate studies. Jennifer has placed her own education and life on hold for me, while I finished my degree. I cannot thank her enough for everything she has done to support me outside of the lab.

DEDICATION

This thesis is dedicated to three people:

My wife Jennifer, without her love and support I would have never finished graduate school.

and

My grandmother, Mary Huys, and brother-in-law, Mark Wilson, both who helped shape the person I am today; and who I sadly lost during my time in graduate school.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	Amino Acid
Ago	Argonaute
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ARE	AU-Rich Element
BMV	Brome Mosaic Virus
CAT-1	Cationic Amino Acid Transporter
CstF	Cleavage Stimulation Factor
CXCR4	C-X-C Chemokine Receptor Type 4
CypA	Cyclophilin A
DAA	Direct-Acting Antiviral
DENV-2	Dengue Virus-2
DGCR8	DiGeorge Syndrome Critical Region Gene 8
ER	Endoplasmic Reticulum
EJC	Exon Junction Complex
EMCV	Encephalomyocarditis Virus
Fluc	Firefly Luciferase
HSPG	Heparin Sulphate Proteoglycans
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIF-1α	Hypoxia Inducible Factor 1α
hnRNP	Hetrogenous Ribonuclear Protein
hr15-LOX	Human Reticulocyte 15-Lipoxygenase
HIV	Human Immunodeficiency Virus
hVAP-A	Human Vesicle-Associated Membrane Protein-Associated Protein A
IMP-1	Insulin-Like Growth Factor 2 mRNA Binding Protein 1
IFNα	Interferon α

IAV	Influenza-A Virus
IRES	Internal Ribosome Entry Site
ITAFs	IRES Trans-Acting Factors
JCV	JC Virus
JEV	Japanese Encephalitis Virus
JFH-1	Japanese fulminant Hepatitis-1
KSRP	K Homology Splicing Regulatory Protein
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LNA	Locked Nucleic-Acid
LVP	Lipoviroparticle
miRNA	Micro RNA
miR-122	Mirco RNA 122
mRNP	mRNA Ribonucleoprotein
MW	Membranous Web
N	Nucleocapsid
NS-1	Non-Structural Protein-1
NSAP1	NS1 associated protein 1
NPC1L1	Niemann-Pick C1-Like 1
NP	Nucleoprotein
ORF	Open Reading Frame
PABP	Poly(A)-Binding Protein
PBS	Phosphate-Buffered Saline
P-body	Processing Body
PV	Poliovirus
Pri-miRNA	Primary miRNA
Pre-miRNA	Precurssor miRNA
PFV	Prototype Foamy Virus
Rap55	RNA-Associate Protein 55
RLuc	Renilla Luciferase
RISC	RNA-Induced Silencing Complex

RNP	Ribonucleoprotein
RRE	Rev-Response Element
SGR	Sub-genomic Replicon
SHAPE	Selective 2'Hydroxyl Acylation Analyzed by Primer Extension
siRNA	Silencing RNA
SL	Stem-loop
SR-B1	Scavenger Receptor Class B Type 1
SVR	Sustained Virological Response
TfR1	Transferrin Receptor 1
TRBP	HIV-1 Transactivating Response RNA-Binding Protein
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low-Density Lipoprotein
WNV	West Nile Virus
WT	Wild-type

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Hepatitis C virus introduction

1.1.1 Clinical characteristics

Hepatitis C virus (HCV) is a global health concern. There are roughly 150 million people currently infected with the virus (1), with an estimated 250 thousand being Canadians (2). There are six genotypes of the virus distributed throughout the world, with genotype 1 being most prominent in North America (3).

The virus is spread from person to person through infected blood or blood derived bodily fluid (4). The commonly accepted modes of transmission are through blood transfusions with infected blood, sharing needles, accidental needle sticks, sexual contact and mother to child transmission during childbirth (4-6). Once infected with the virus, patients either spontaneously clear the infection and suffer few, if any symptoms, or develop a chronic hepatitis infection. Unfortunately patients develop chronic hepatitis roughly 70% of the time. Those who suffer from chronic hepatitis are at risk of developing liver cirrhosis, hepatocellular carcinoma (HCC) and death (1). Due to the level of cirrhosis associated with HCV, its infections are the leading cause for liver transplantation in North America (7).

Currently, there is no vaccine to prevent HCV infections. Traditionally, the treatment for those infected with the virus was a combination of pegylated IFN-α and ribavirin (8). This combination treatment is effective roughly 70 - 80% of the time for genotype 2 and 3 but only 50% effective against genotype 1 (9). Fortunately for patients infected with genotype 1, there have recently been new direct-acting antiviral agents (DAAs) licensed for use. In combination with IFN-α and ribavirin these DAA have been demonstrated to be 60%-90% effective at clearing the virus from patients infected with genotype 1 (10, 11).

1.1.2 Molecular characteristics

HCV is an enveloped positive strand RNA virus belonging to the *Flaviviridae* family. Its genome is 9.6kb in length and encodes a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR). An internal ribosome entry site (IRES) resides within the 5' UTR and directs cap-independent translation of the HCV polyprotein, which is cleaved into ten individual proteins by both host, (endoplasmic reticulum signal peptidase and single peptide peptidase) and viral proteases. The 10 individual proteins are termed core, E1, E2, p7, NS2 through NS5 (12, 13) (Fig. 1.1).

1.1.2.1 Structural proteins

Structural proteins core, E1, and E2 are derived from the N-terminal portion of the HCV polyprotein. Core protein forms the HCV nucleocapsid and binds to and surrounds the genomic RNA in the HCV virion. Core protein is composed of two domains. The first domain is predicted to interact with RNA while the second domain interacts with lipids (14). Core proteins' ability to bind RNA and lipids has led to it being associated with many host and viral proteins (15) but its primary role is as the HCV capsid protein, and it is predicted to play a large role in HCV virion assembly (16, 17).

The HCV envelope proteins E1 and E2 mediate virion attachment and entry into the cell. Both E1 and E2 proteins are ER anchored prior to incorporation into the viral particle. E2 is recognized by both CD81 and scavenger receptor class B type 1 (SR-B1) and plays a pivotal role in HCV entry (18).

1.1.2.2 Non-structural proteins

Non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (12, 13) are cleaved from the C-terminal portion of the HCV polyprotein. P7 is believed to form an ion channel and has been demonstrated to be important for regulating the

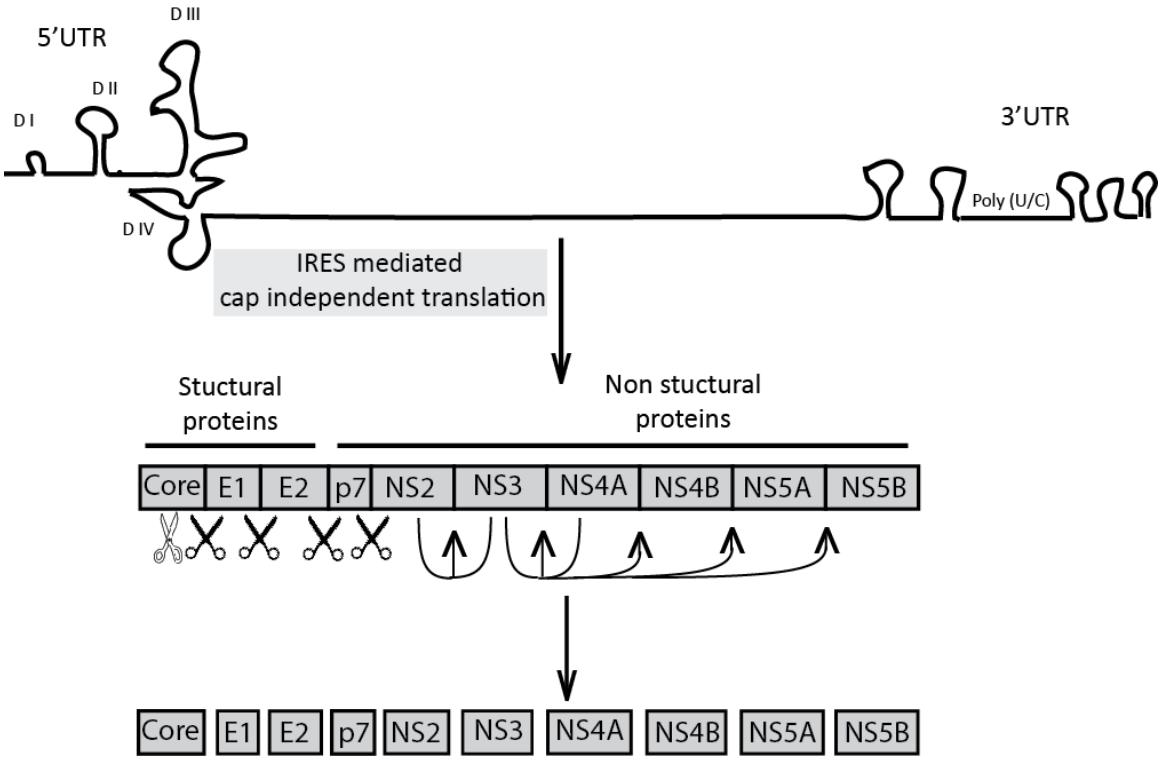

Figure 1.1 HCV genome, translation and processing. A schematic diagram of the HCV genome, highlighting the complexity of the 5' and 3' UTR. The polyprotein that is translated from the HCV genome, and the cleavage sites within the polyprotein that results in the individual HCV proteins. The hollow scissors represent the cleave of core by single peptide peptidase, the solid scissors represent cleave events performed by ER singnal peptidase and arrows denote sites that are cleaved by HCV proteases, either NS2 or NS3.

pH of the virion during egress from the cell (19). P7 also interacts with NS2, and core, a step proposed to be important for virus assembly (20).

During translation of viral proteins, NS2 works as a protease to cleave itself from NS3 (21). It then recruits viral, and possibly host proteins, required for HCV to assemble and then bud into the ER lumen (22).

NS3 is both a DEAD-box helicase, a type of helicase protein that will be discussed in depth in section 1.6 (13), and a protease. NS3 promotes its own cleavage from NS2 in the polyprotein, and along with its co-factor NS4A is the protease responsible for cleaving much of the HCV polyprotein (23, 24).

In addition to its role as a cofactor for NS3's protease and helicase activity (25), NS4A's association with NS3 allows the complex to be anchored to the ER, a process that is thought to enable the two proteins to augment HCV assembly (24, 26).

NS4B is a hydrophobic protein with an important, but poorly understood role in the HCV life cycle. NS4B induces the formation of the HCV infection induced membranous web (MW), the site of the HCV replication complex and HCV RNA replication. Thus, an interaction between NS4B and the replicating RNA cannot be ruled out, and perhaps plays a role in HCV replication (27). Others have also demonstrated that a mutated form of NS4B can increase HCV particle production while not affecting replication, suggesting a role in virus assembly (28).

NS5A has been demonstrated to affect HCV translation, replication and assembly. How NS5A participates in many of these activities is not known, but NS5A exists in two states, a hyperphosphorylated state and a hypophosphorylated state. Hypophosphoyrlated NS5A is thought to promote RNA replication while hyperphosphorylated NS5A suppresses replication (29-31). NS5A's ability to localize to lipids and interact with core is thought to be essential for the virus' life cycle (12, 32, 33).

NS5B is an RNA dependent RNA polymerase and as such is responsible for the synthesizing of both the positive and negative RNA strands of HCV. There are also studies that indicate NS5B is required for HCV assembly but its exact role is unknown (18, 34).

1.1.3 HCV life cycle

An overview of the entire HCV life cycle is summarised in Figure 1.2 and a summary of how HCV attaches and enters a hepatocyte is illustrated in Figure 1.3.

1.1.3.1 Attachment and entry

Infectious HCV virus particles are assembled in a way that makes them similar to very-low density lipoprotein (VLDL) or low-density lipoprotein (LDL). Interestingly, lower density HCV particles are more infectious to human hepatocytes. Similarities between the appearance of HCV particles in comparison to VLDLs and LDLs have led to HCV particles being named lipoviroparticles (LVPs). The composition of the LVP allows for a myriad of interactions with host receptors. As such, many receptors including, heparin sulphate proteoglycans (HSPG), the low-density lipoprotein receptor (LDLR), SR-B1, and Niemann-Pick C1-like 1 (NPC1L1) have all been identified to influence HCV attachment and subsequent steps required for entry by clathrin-mediated endocytosis (35).

The first interaction a LVP has is with HSPG on the host cell. HSPG is thought to bind apolipoprotein E (ApoE) moieties within the LVP and allows attachment of the virus but does not assists in cell entry (36). LDLR has also been suggested to assist in a similar fashion but it is not essential. In fact its interaction with LVPs has also been demonstrated to be detrimental to the virion, thus the importance of its interaction is not fully understood (37, 38). After association with HSPG and/or LDLR, the LVP associates with SR-B1. The ability of SR-B1 to assist in attachment depends on the composition of the LVP and its access to HCV E2 (39-41). SR-B1 is dispensable for virion attachment but is required for virus entry. Virion interaction with SR-B1 promotes E2 binding to the tetraspanin protein CD81, and virion entry requires SR-B1's ability to transfer lipids from the lipoprotein to the cellular membrane, not its interaction with E2, since a mutation in SR-B1 that inhibits its ability to transfer lipids does not inhibit HCV attachment, but prevents viral entry (41, 42).

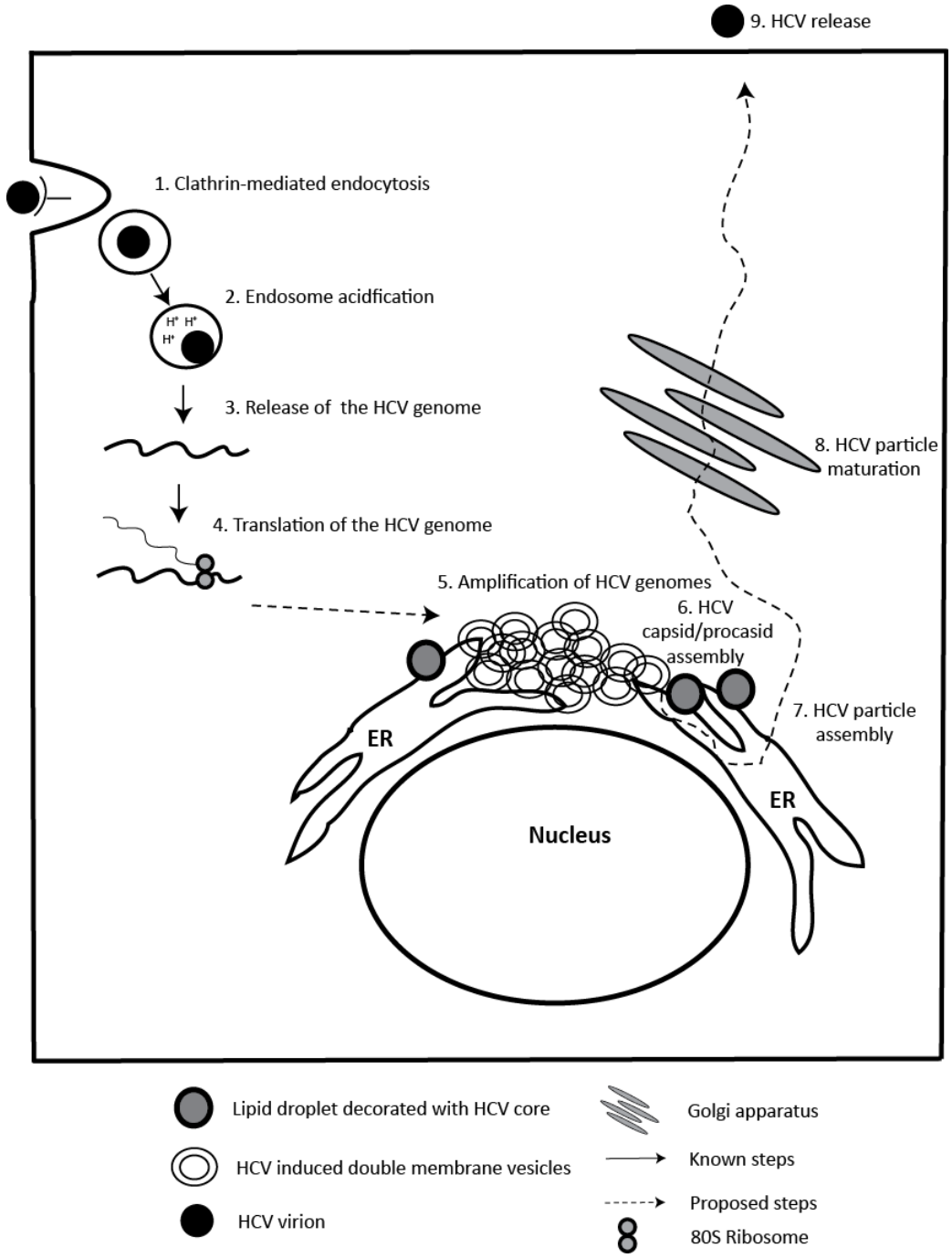

Figure 1.2 HCV life cycle. Step 1: HCV enters the cell by clathrin-mediated endocytosis. Step 2 & 3: HCV fuses with the endosome through endosome acidification, which releases the HCV genome into the cytoplasm. Step 4: The HCV genome translates its proteins. Step 5: HCV proteins induce the formation of a membranous web from ER membrane, and viral RNA replicates within them. Step 6: The capsid/pro-capsid assembles in close proximity to core decorated lipid droplets and buds into the ER. Step 7: HCV acquires its envelope and begins to associate with lipids. Step 8: HCV is transported through the Golgi and enters the secretory system in a similar fashion to VLDLs. Step 9: The HCV virion is released from the cell.

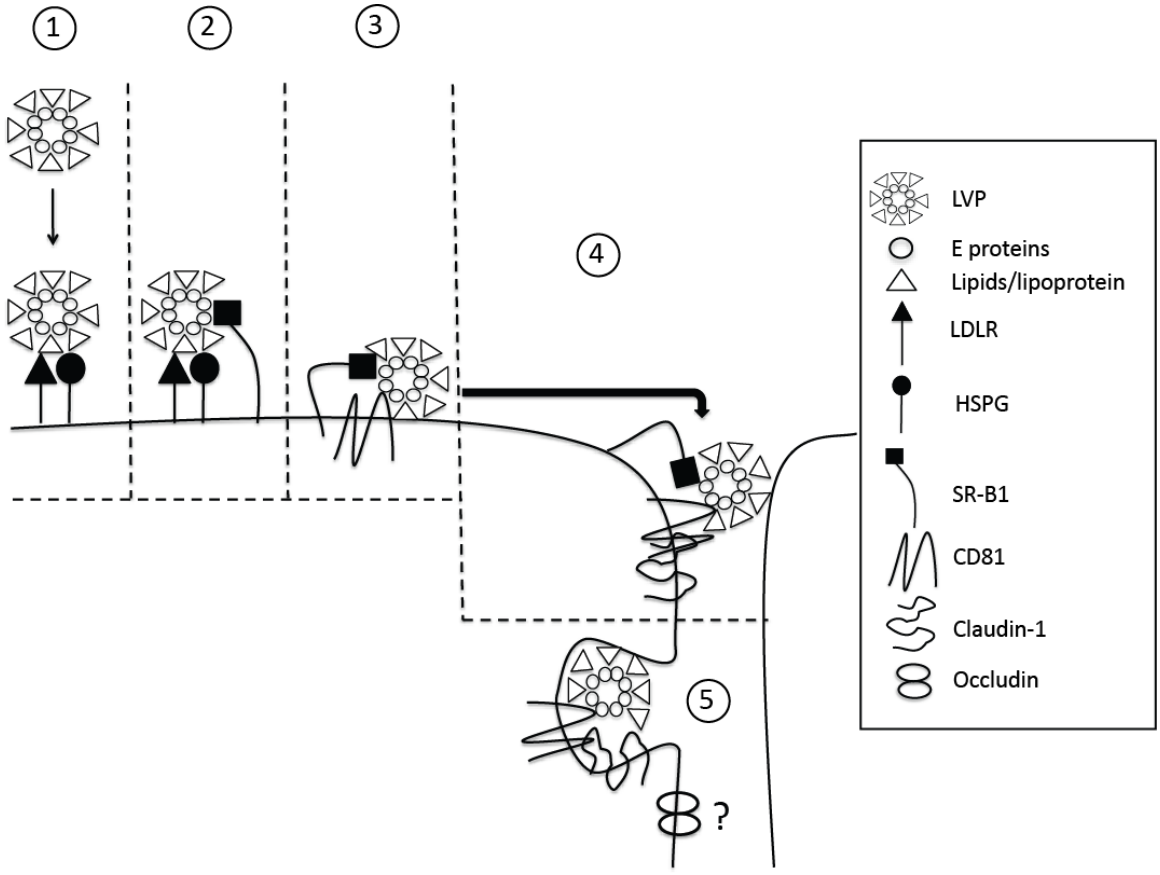

Figure 1.3 HCV entry into hepatocytes. Step 1: The LVP attached to HSPG and/or LDR. Step 2: SR-B1 interacts with VLP and E2. Step 3: CD81 binds E2. Step 4: CD81 and the LVP translocation to the tight junction between hepatocytes were CD81 interacts with claudin-1. Step 5: Clathrin-mediated endocytosis of LVP along with CD81 and probably claudin-1

CD81 binding is important for two reasons: it prepares HCV for fusion with the endosome, (43) and stimulates its translocation to hepatocyte tight junctions (44). Within the tight junctions CD81 interacts with the tight junction proteins claudin-1 and occludin (45, 46). Claudin-1 and occludin have no known physical interaction with the LVP and are thus considered co-receptor proteins. How occludin aids in HCV entry is not known but it does not involve attachment (47). At some point after HCV interacts with CD81, the transferrin receptor 1 (TfR1) assists in HCV entry by an as yet unknown mechanism (48).

Recently the Niemann-Pick C1-like 1 (NPC1L1) has been identified as a receptor required for HCV entry. There is no evidence that NPC1L1 interacts directly with HCV, but it appears to interact with cholesterol on the LVP (49). Lindenbach and Rice in a recent review speculated that NPC1L1 might help modify the LVP to further induce the interaction between CD81 and E2 (18) as it prepares HCV for fusion with the endosome.

Claudin-1's role in HCV entry is also uncharacterized but data suggest that the HCV virion-CD81 complex, and claudin-1 induce clathrin-mediated endocytosis (50, 51). After clathrin-mediate endocytosis, HCV fusion with the endosome is triggered by endosome acidification and the genome is released into the cytoplasm were it can begin to translate proteins from its genome (43, 52)

1.1.3.2 Translation

Once access to the cytosol is gained the HCV genome serves as a template for translation. Mentioned briefly in 1.1.2, HCV utilizes an IRES located in the 5'UTR for cap-independent translation. RNA in the 5'UTR forms a structured IRES RNA element, which is able to recruit translation initiation proteins and ribosomes independent from the cap binding proteins to initiate translation.

The HCV 5'UTR contains four domains, domains I to IV, which are highly conserved among genotypes. Domains II, III, IV, and sequences past the UTR into the core coding sequence comprise the IRES. Domain III is responsible for recruiting the 40S ribosome and all three domains act to retain the 40S ribosome and place it

in the proper orientation with respect to the AUG start codon. Once the ribosome is recruited and retained, the cellular initiation factors are recruited. eIF3 is recruited by domain III and associates with the 40S ribosome. This association allows the recruitment of the ternary complex eIF2-GTP-tRNA^{met}, which upon phosphorylation by eIF5 leads to the addition of the 60S ribosome and the formation of the translational competent 80s ribosome complex (53-55).

HCV translation is also influenced by *cis*-acting elements outside of the IRES. Immediately down stream of the IRES in the core coding sequence there are numerous secondary structures, which have been demonstrated to up-regulate and down-regulate HCV translation. As summarized in a recent review by Hoffman and Liu, the influence of the core region on HCV translation is suggested to be mediated by viral and/or host protein binding or long-range RNA-RNA interactions between the core region and other parts of the HCV genomic RNA (54). Additionally, a stem-loop present in the NS5B coding region of HCV has been demonstrated to enhance HCV translation by interacting with the IRES through domain III (56).

Similar to the 5'UTR, the 3'UTR is also divided into distinct domains. The three domains of the 3'UTR are the variable domain, the poly (U/C) domain, and X-tail domain. All three domains up-regulate HCV translation, presumably through direct RNA-RNA interaction with the 5'UTR, or possibly by proteins attached to the 3'UTR stimulating HCV translation (57). The interaction of the 5'UTR with the 3'UTR also indicates that HCV translation probably occurs in a closed-loop orientation, like that of cap-dependent translation.

HCV IRES function is also influenced by *trans*-acting host proteins, known as ITAFs (IRES *trans*-acting factors). These proteins include the La autoantigen, also called the La protein, mice minute virus NS1 associated protein 1 (NSAP1), insulin-like growth factor 2 mRNA binding protein 1 (IMP-1), LSm1-7, heterogeneous ribonuclear protein D and L (hnRNP D and hnRNP L), that bind directly to, or in close proximity, with the IRES, and promote HCV translation (58-61). Conversely, Gemin5 down regulates translation by interacting with domain III of the IRES (62). Interestingly, many of the ITAFs demonstrate the ability to interact with each other suggesting a complex role in coordinating HCV translation (55, 60, 63, 64).

HCV proteins represent another set of ITAFs, however the roles of the individual HCV proteins are controversial. Many reports have indicated that the same proteins can increase, decrease or have no effect on HCV translation (54, 65). The development of better culture systems should provide a more definitive understanding of the effects the individual HCV proteins have on HCV translation in the near future.

Lastly, certain miRNAs have been identified that affect HCV translation. The miRNA miR-199a*, miR-196 and miR-122 have all been identified to affect HCV translation and their role in HCV life cycle will be discussed in depth elsewhere.

1.1.3.3 Replication

As HCV is a positive sense RNA virus, its genome acts as a template for both protein translation (which occurs in the 5' to 3' direction) and RNA replication (which proceeds in the 3' to 5' direction). Thus, at some point the HCV genome must cease translating, and initiate replication. How HCV regulates the switch from translation to replication is not yet known, and the process by which HCV replicates is still poorly understood. Limiting our understanding of HCV replication is the difficulty in separating translation from replication without abolishing the virus life cycle. Replication appears to function in *cis,* by proteins derived from the genome, so thus far methods to study replication in isolation of translation have not been developed. Another limiting factor is the inability to *trans*-complement viral components involved in HCV genome replication. Thus, it is difficult to determine what individual viral proteins are doing. Most studies must thus rely on microscopic techniques to observe protein location and replication induced structures in HCV infected cells.

During the HCV life cycle, HCV protein expression causes the appearance of a MW in the cytosol. The MW is derived from the endoplasmic reticulum (ER) membrane and is thought to be the site of HCV replication (66, 67). The topology of the MW that HCV utilizes for replication is still under debate. Recent evidence suggests that replication occurs inside or on the outer surface of infection induced

double membrane and/or multiple membrane vesicles (66). The inability to demonstrate actively replicating HCV RNA due to the limitations of the current methods prevents definitive answers on where HCV replicates in the MW.

RNA replication occurs through a RNA negative strand intermediate. Progeny RNA synthesis is carried out by the viral RNA dependent RNA polymerase, NS5B. NS5B can generate both the positive and the negative strand HCV RNA *in-vitro* (68) but this synthesis requires self-priming in the presence of large amount of nucleotides. *In-vivo*, it is likely that NS5B relies on viral and host proteins to assist in priming and initiation of RNA synthesis, however this mechanism has not been elucidated.

The ability to construct viable sub-genomic replicons indicates that the non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B are sufficient for HCV RNA amplification. Using HCV replicons and genetic screens, several host proteins associated with HCV replication have been identified and have been described in detail elsewhere (69). However, I will describe a few key examples of HCV virus-host interaction. Human vesicle-associated membrane protein-associated protein A (hVAP-A), a protein involved in intracellular vesicle trafficking, interacts with the hypophosphorylated form of NS5A and promotes RNA replication (70). In addition, the hVAP-A interaction and phosphorylation of NS5A has been suggested to regulate the switch between HCV translation and replication (71, 72). In a second example Cyclophilin A (CypA), a cellular chaperone protein, interacts with NS5A to promote HCV replication. CypA is thought to promote HCV replication by altering the conformation of NS5A (73), but its precise role in HCV replication remains to be determined (69, 74). Interestingly, CypA inhibitors such as alisporivir, are emerging as promising anti-HCV therapeutics (9, 75).

Similar to HCV translation, *cis*-acting elements in the HCV genome have been demonstrated to influence HCV replication. As discussed previously, the 3'UTR of HCV is divided into three regions (the variable domain, the poly (U/C) domain, and X-tail domaim), and not surprisingly given that negative strand RNA replication begins at the 3' end, each region has been shown to be important for HCV replication. The X-tail domain contains 3 highly conserved stem-loops and

mutational analysis of the stem-loops indicated that both the sequences and structures of the stem-loops are required for HCV replication (69, 76). The second stem-loop has been demonstrated to interact with a stem-loop located in the NS5B coding region (77). This interaction is essential for HCV replication, and although the stem-loop in the X-tail domain cannot be moved, the stem-loop in the NS5B coding region can be relocated to the 3'UTR and replication can still occur. Interestingly, the last base in the 3' end of HCV participates in stem-loop base paring thus limiting its access by NS5B. This stem-loop is thought to protect the RNA from degradation, but also indicates a level of complexity required for HCV to replicate the negative strand.

The poly (U/C) domain is required for HCV replication, and must contain over 26 consecutive uracils for replication to occur (78). The poly U track is thought to bind viral and host proteins, but its exact roll in replication is not fully understood. The variable region of the 3'UTR is not essential for HCV replication but alterations to it leads to a decrease in HCV replication, indicating it provides a function for replication (79).

The 3'UTR of the negative strand is the complement of the 5'UTR and is thus also important for positive strand genome synthesis. Not much is known regarding how the 3'end of the negative strand induces RNA synthesis, but it is proposed to be simpler because unlike the 3'end of the positive strand, the last base is not involved in a stem-loop structure and therefore theoretically is easier to prime for initiation and elongation (80). The secondary structure of the minus strand 3' end has also been predicted to form 5 stem-loops within the initial 250 base-pairs (81-84). Utilizing bi-cistronic constructs, the first three stem-loops were demonstrated to be required for positive strand synthesis, and the other loops, although not required, also contribute (84, 85).

1.1.3.4 Assembly and release

Following HCV genome replication in the MW, some of its replicated genomes are assembled into new virions. The assembly and release of the virus is thought to

occur in parallel with the formation and release of lipoproteins and is not cytolytic. Again, the definitive mechanism by which HCV virions are assembled and released is poorly understood but experimental evidence sheds some light on assembly and release.

Virion assembly appears to be initiated by an interaction between NS5A and core on lipid droplets (86). The HCV nucleocapsid protein, core, associates with cytosolic lipid droplets after it is synthesized (14). HCV virion assembly is initiated concurrently with the disassociation of core from the lipid droplets. An interaction between NS5A and core may facilitate the disassociation between core and lipid droplets (32, 87). This step is thought to bridge the replication complexes and assembly since NS5A is associated with replication complexes, and thus HCV genomes, and perhaps acts as a signal to initiate assembly. In the next step of virion assembly, HCV leaves the MW and buds into the ER. NS2 is thought to orchestrate this process by recruiting the ER anchored HCV envelope proteins E1 and E2, along with NS3-4, and p7 to the core-lipid-HCV RNA-NS5A complex (88-90). Although the non-structural proteins are not associated with the virion particle after release, they are required for assembly and possibly egress. These interactions are thought to result in viral RNA being transported in association with core to bud into the ER (18, 34).

Once in the ER, HCV virions enter a secretory pathway similar to that used by VLDLs. During this process HCV likely interacts with lipoproteins, however HCV does not appear to be assimilated into VLDLs because VLDLs require ApoB, a protein that can be present on, but is not required for HCV particles; thus, how and when HCV requires its lipoproteins is not fully understood (91, 92). However, when HCV acquires its lipoproteins it requires ApoE, as it is essential to generate mature infectious particles (92). HCV is then released into the cytoplasm, or alternatively can directly infect neighbouring cells using a process that does not require release of the particle (93).

1.2 Hepatitis C virus life cycle and miRNAs

Cellular miRNAs are endogenously expressed short double stranded RNAs that associate with mRNAs and modulate their translation and stability. Human cells express over 1000 miRNAs, and they are estimated to regulate at least one third of the total mRNAs expressed (94). The HCV life cycle is influenced directly and indirectly by cellular miRNAs. Several miRNAs that affect HCV infection and pathogenesis have been reviewed by Thibault *et al*. (95). This thesis will focus extensively on the interaction between HCV and the microRNA-122 (miR-122) with consideration to potential host accessory proteins that are involved in their relationship.

1.2.1 miR-122

1.2.1.1 Cellular function

The miRNA miR-122 is abundantly expressed in the liver and is estimated to constitute 70% of all the total miRNAs expressed in the mammalian liver, with roughly 135 000 copies per cell (96). miR-122 is 22 nucleotides long and is derived from the 5' end of the hcr mRNA, a non-coding gene. Interestingly, miR-122 has been demonstrated to regulate the expression of cationic amino acid transporter 1 (CAT-1). Under normal cell conditions miR-122 sequesters CAT-1 in p-bodies, but during times of stress, such as hepatocyte re-generation, miR-122 repression is released, and CAT-1 is actively translated (97). In the context of the liver, miR-122 regulates lipid and cholesterol metabolism, hepatocyte differentiation, circadian regulation, and iron homeostasis (98, 99). Recently, several targets within these pathways have been demonstrated to directly interact with miR-122 (100-102). However, miR-122 regulation cannot explain the outcome of the effects on all these pathways suggesting that many miR-122 targeted genes are yet to be identified. Short-term inactivation of miR-122 does not appear to be detrimental, and may in fact be beneficial, as it has been demonstrated to lower serum cholesterol (103). However,

miR-122 knockout mice exhibit persistent hepatosteatosis, fibrosis and develope HCC, suggesting that miR-122 has important roles in the liver including as a tumour suppressor (98, 102).

1.2.1.2 Direct interaction with the HCV genome

miR-122 has an unconventional relationship with HCV. The conventional interaction between a miRNA and its target mRNA leads to suppressed protein expression. In the case of miR-122 and HCV, the interaction promotes expression, and virus replication rather than acting as a suppressor. The relationship between HCV and miR-122 is also unconventional in that miR-122 interacts with the 5' end of the HCV genome instead of the customary 3' end (Fig. 1.4). Jopling *et al.* observed that in order for HCV to replicate, it requires direct association of miR-122 with two binding sites in the HCV 5'UTR. This was demonstrated by the fact HCV contains two sites that are separated by a highly conserved 8-9 nucleotides, which are complementary to miR-122 seed sequence (a 7-8 nucleotide span in the 5' end of the miR-122 required for miRNA suppression activity) in its 5'UTR. Mutations to these sites rendered the virus incapable of replicating unless a synthetic miR-122 that contained complimentary mutations that reinstate binding was added to the experiment (104, 105). These experiments demonstrated that miR-122 was required for HCV replication, and its requirement is through a direct nucleotide interaction between HCV genomic RNA and miR-122. In addition to miR-122 seed sequence, binding of miR-122 to the HCV genome also required annealing of nucleotides outside of the seed sequence (106). The amount of annealing of the extra nucleotides varies between HCV genotypes (107), however when the miR-122 seed sequence is bound at binding site 1, nucleotides 15 and 16 of miR-122 also interact with the HCV 5' terminus at conserved nucleotides at position 2 and 3 (106, 107) (Fig 1.4). This interaction is thought to give HCV 5'end the appearance of dsRNA and potentially protects it from degradation.

Analysis of HCV genome has revealed two other potential miR-122 binding sites. The third site was identified at the same time as the first two sites and

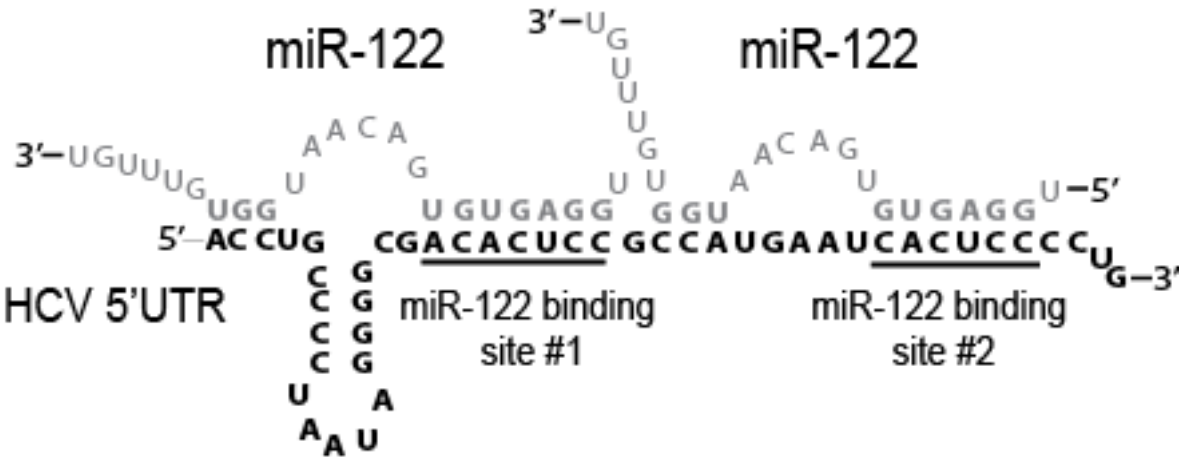

Figure 1.4 miR-122 bound to the 5'UTR of HCV genotype 2a. A schematic diagram of how two molecules of miR-122 bind and interact with the 5'UTR of HCV genotype 2a.

evidence suggests it does not play a role in HCV life cycle (105, 108). The fourth site is present in genotypes 1, 3, 4, and 6 and displays the ability to inhibit HCV replication and translation (109). Since the fourth site is not present on all genotypes, its purpose in HCV replication is unknown but may be important for suppressing HCV during the establishment of a chronic infection in genotypes which encode this fourth miR-122 bindings site.

1.2.1.3 Effects on HCV

Early reports indicated that miR-122 promoted HCV RNA replication but did not influence viral translation (105). Subsequent reports confirmed that HCV can translate without miR-122 binding to HCV 5'UTR, but that miR-122 binding to the 5'UTR promotes viral translation by about 2 fold (108, 110-112). However, in spite of the influence of miR-122 on HCV translation, the increase in translation does not account for HCV dependency on miR-122 for replication. This was demonstrated by comparing replication levels of an HCV mutant, which could not bind miR-122 with an HCV mutant having a modified IRES that attenuated translation to a similar extent as the miR-122 binding-mutant. While replication of the IRES mutant was attenuated, the mutant that could not bind miR-122 exhibited no replication at all (110). This suggests that while miR-122's influence on translation may enhance replication, other roles that promote HCV replication likely exist.

One other role for miR-122-binding is to stabilize the HCV genomic RNA (113-115). miR-122 increases HCV stability by protecting the 5'end of HCV from degradation by the 5' to 3' exonuclease Xrn1 (113, 115). Following knockdown of Xrn1, HCV RNA to which miR-122 cannot bind, displayed the same decay rate and protein expression as HCV RNA in the presence of miR-122 (without Xrn1 knockdown) (113). This observation suggests that miR-122 augments HCV translation solely through protecting the genome from degradation by Xrn-1. However, an HCV mutant that could not bind miR-122 was still unable to replicate even when Xrn1 was knocked down, indicating a secondary role for miR-122 in

promoting HCV replication (113). How miR-122 promotes replication independent from genome stabilization is still unknown.

Although many reports suggest that miR-122 is required for HCV replication, there is evidence that HCV can replicate independently of miR-122. HCV sub-genomic replicons replicate in Hep3B cells which to do not produce detectable levels of miR-122 (116). The observed replication was approximately 100 fold less efficient than miR-122-dependent replication. Similarly, Li *et al.* demonstrated that RNAs having point mutations to the first miR-122 binding site were able to replicate in the presence of a miR-122 antagonist, which rendered wild-type (WT) virus incapable of replicating, suggesting that these RNAs had escaped the requirement for miR-122 (117). Although the replication of these viruses in the presence of miR-122 was a fraction of WT virus, the data suggest that miR-122 greatly enhances HCV replication, but may not be absolutely required for it.

Another possibility is that miR-122 may regulate HCV stability, translation and/or replication by modifying the secondary structure of HCV genomic RNA. Several *in-vitro* studies have been conducted to analyze RNA conformational changes miR-122 can cause in the 5'UTR. One study indicated that miR-122 binding affected a long range RNA-RNA interaction that may function as a switch between HCV replication and translation. The interaction was predicted to occur between the 5'UTR and a region within core protein's coding sequence (118). However, since this region of core is not present in HCV sub-genomic replicons whose translation and replication are also stimulated by miR-122, the biological relevance of this potential interaction is unknown (116). Two other studies analyzed changes to the conformation of the HCV 5' UTR by using selective 2' hydroxyl acylation analyzed by primer extension (SHAPE) analysis. SHAPE analysis works by adding bulky adducts to the ribose 2' hydroxyl group of RNA, which blocks elongation by reverse transcriptase at the nucleotide. The availability of the 2' hydroxyl group varies depending on whether the nucleotide is involved in RNA secondary interaction. Thus, after primer extension by reverse transcriptase, the product can be run on a gel to determine the availability of the nucleotide based on the size and intensity of fragments visualized (119). In these studies, miR-122's affinity for the second site

was greater than for the first, and binding to the second site was not required for interactions with the nucleotides outside of the seed sequence (115, 120). In addition, the interaction of miR-122 with the second miR-122 binding site caused structural modification to the 5'UTR, outside of the miR-122 binding site region, and within the HCV IRES (115). While these modifications could promote or inhibit translation and/or replication, the biological relevance of the findings *in-vivo* are unknown and require further analysis, since these studies were conducted *in-vitro* with single stranded miR-122 in the absent of cellular proteins.

1.3 HCV replication and miRNA proteins

Given the requirement for miR-122 to bind to the HCV genome, it is not surprising that many of the proteins involved in miRNA suppression have been linked to HCV replication, since a cascade of host proteins are required for miRNA biogenesis, processing, and suppression activity. Further evidence for the involvement of host proteins to process miR-122 before it can be used to augment HCV replication comes from the observation that single stranded synthetic miR-122 can not promote HCV replication suggesting that more than simple RNA-RNA annealing is required (112).

1.3.1 miRNA biogenesis proteins required for miR-122 augmentation of HCV replication

1.3.1.1 miRNA biogensis

Biogenesis of miRNA commences in the nucleus as primary miRNA (pri-miRNA), naturally occurring hairpins within noncoding RNA transcripts (121). The microprocessor complex (MPC) cleaves the individual pri-miRNA hairpin from the transcript to form precursor-miRNAs (pre-miRNA) but pre-miRNAs can also be derived from exon and introns of coding RNA. The MPC is composed of the RNase III enzyme Drosha in a complex with DiGeorge syndrome critical region gene 8

(DGCR8) (122). The cleavage of the pri-miRNA occurs when DGCR8 recognizes the hairpin and the single stranded RNA next to the hairpin, which leads to the recognition and Drosha cleavage to generate the pre-miRNA hairpin with a two-nucleotide overhang (123).

Once processed, the pre-miRNA is exported from the nucleus into the cytoplasm. Translocation from the nucleus occurs via the Exportin 5-RanGTP process (124). Exportin 5 recognizes and binds the hairpin structure, and not only facilitates transport of the pre-miRNA but also stabilizes the hairpin structure (125). The pre-miRNA is released into the cytoplasm by the hydrolysis of RanGTP (125). Once in the cytoplasm, Dicer further processes the pre-miRNA to a mature miRNA. Dicer binds to the pre-miRNA and its RNase III activity removes the loop portion of the hairpin to leave a 22 nucleotide double stranded RNA having a 2 nucleotide overhang at each 3' end (126, 127).

Following cleavage, Dicer retains the mature miRNA and with the help of HIV-1 transactivating response RNA-binding protein (TRBP) and/or PACT is able to interact with, and transfer the miRNA to an argonaute protein. Argonaute proteins are key proteins in the complex that mediates miRNA gene silencing and is the core of a miRNA-protein complex called the RNA-induced silencing complex (RISC) (128-130).

1.3.1.2 HCV and the biogenesis of miR-122

During the HCV life cycle, Dicer appears to be required to process pre-miR-122 to miR-122. Knocking down Dicer inhibits HCV replication, but its effect can be reversed by supplying the cells with synthetic mature miR-122, indicating that HCV requires Dicer to process miR-122 for its use (112), but suggests that Dicer is dispensable for miR-122 loading into RISC. Similarly, TRBP has also been implicated in miRNA biogenesis, and its knockdown leads to decreased HCV replication. Contrary to Dicer, the supplementation of miR-122 does not fully restore HCV replication in TRBP depleted cells suggesting a secondary role of TRBP in the HCV life cycle (112), possibly in loading miR-122 into RISC.

1.3.2 miRNA gene suppression proteins required for miR-122 augmentation of the HCV life cycle

1.3.2.1 miRNA gene suppression: strand selection, incorporation into RISC, and mRNA suppression

Human cells have four argonaute (Ago) proteins (Ago1, 2, 3 and 4). The Ago proteins play a pivotal role in miRNA gene suppression and are responsible for selecting the strand of the double stranded miRNA that will be retained, and therefore which mRNA sequence will be targeted. Once a miRNA has been transferred to an Ago protein it undergoes strand selection. Strand selection is based on base-pairing thermodynamic stability along the miRNA duplex, with the strand having less stable base pairing at the 5' end being retained and denoted as the guide strand, while the other strand, the passenger strand, is discarded (131). The single stranded RNA loaded Ago combines with GW-182 to form a mature RISC capable of suppressing gene expression (132).

Mature RISC utilizes the sequence of the guide strand to bind to the 3'UTR of mRNA. If the binding is 100% complementary between the mRNA and guide strand and the Ago protein participating in the RISC is Ago2, then the transcript can be cleaved at the middle of the guide strand, resulting in the silencing of that mRNA (133). If the binding is not 100% complementary, or the RISC is composed of one of the other three Ago isoforms, then translation can be temporarily stalled, leading to the degradation of the transcript or halting translation for subsequent re-activation. Silencing requires the host protein GW-182. Ago proteins interact with GW-182 and GW-182 is predicted to interact with the poly-A binding protein and impede translation by preventing the circularization of the transcript (132). GW-182 also recruits de-adenylating and de-capping enzymes, which leads to de-adenylation and de-capping of the transcript resulting in 5' to 3' degradation of the transcript by Xrn1 (94, 134, 135).

1.3.2.2 miRNA suppression proteins, miR-122, and HCV

miR-122 recognizes and binds HCV as part of RISC, or a RISC like complex. This was supported by the fact that the Ago proteins are required for efficient HCV replication, and for miR-122 to promote HCV replication (136). Early evidence suggested that all four Ago proteins influence HCV replication, but recent observations suggest that only Ago2 has a significant effect on HCV (114). Knockdown of Ago2 leads to a decrease in HCV replication and translation, which is directly related to the dependency of HCV on miR-122 (111). Ago2 in combination with miR-122 has been demonstrated to bind to the 5'UTR of HCV, and this association stabilizes the genome and promotes viral translation (114, 137). In the absence of Ago2, miR-122 cannot enhance HCV stability, indicating that Ago2 is required for the interaction between miR-122 and HCV (114). At this time it is unknown whether Ago2 is simply required for delivery of miR-122 to the HCV genome or if Ago2 and/or GW-182 participates in the mechanism of action of miR-122. A role GW-182 in HCV replication has been observed, but conflicting activities have been reported (138-141). GW-182 is transcribed as three different isoform, two of which have been demonstrated to affect HCV translation (140). How GW-182 affects HCV translation is unknown, but given its association with Ago2, it seems plausible that GW-182 assists in miR-122 binding to the 5'UTR, but this remains to be confirmed. However, others have reported that GW-182 had no effect on HCV replication or translation (139, 141) thus, the effect of GW-182 on HCV replication requires further research in order to determine what effects, if any, GW-182 may have on HCV and miR-122 augmentation.

1.4 The role of p-bodies in miRNA suppression and miR-122 augmentation of HCV

Many of the proteins associated with miRNA directed mRNA degradation are found within special cellular compartments called processing bodies (p-bodies) and

thus miRNA suppressed transcripts are thought to be stored and degraded in p-bodies (97).

1.4.1 Processing bodies (p-bodies)

1.4.1.1 Composition and function of p-bodies

P-bodies are sites of mRNA degradation and storage (Fig. 1.5). How p-bodies are formed is not well understood but many of the proteins found in p-bodies are able to associate with one another, and potentially explains how p-bodies assemble (142, 143). They are transient compartments, whose size and abundance depends on cellular translation conditions. For example, when cells are actively proliferating and therefore actively generating mRNAs, p-bodies are more abundant than in quiescent cells. In addition, when cellular translation is inhibited by drugs or cellular stress, p-body abundance increases to accommodate storage and degradation of untranslated mRNAs (97, 143-147).

There are many proteins that have been demonstrated to be associated with p-bodies, and the majority have also been linked to miRNA silencing and mRNA degradation. These include the RISC associated Ago (148) and GW-182 proteins (132), as well as the de-capping enzymes DCP1 and DCP2, de-capping associating proteins LSm1-7, 5' to 3' exonuclease Xrn1 (149), and the RNA helicase DDX6 (150). Thus, miRNAs may target mRNAs to p-bodies for storage or degradation and may be a key mechanism by which miRNAs silence protein expression. Knocking down many of the resident p-body proteins results in the disappearance of p-bodies, but the disappearance of p-bodies does not necessarily abolish translation repression (151-153). Thus, while some of the p-body proteins are required for miRNA silencing, the presence of microscopically visible p-bodies is not essential. Therefore it is believed that the major role of p-bodies is to degrade transcripts but they may not physically repress translation. This view is supported by the fact that p-bodies do not form unless there are stalled mRNAs in the cytoplasm (154, 155). However, there is evidence that mRNAs can leave p-body structures and re-enter

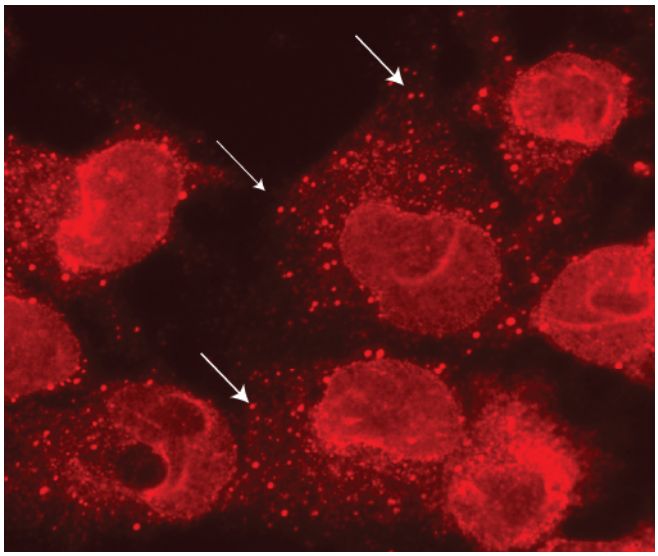

Figure 1.5 P-bodies in Huh7.5 cells. P-bodies were detected in Huh7.5 cells utilizing a polyclonal antibody for the resident p-body protein Ge-1. The white arrows indicate a few of the numerous p-bodies present within the cells.

active translation (97) suggesting that p-body structures, in addition to being sites of degradation may represent storage areas of untranslated mRNAs. How mRNAs are selected for degradation or to re-enter active translation is unknown, but one could imagine it might depend on the proteins associated with the mRNAs.

1.4.2 P-body proteins and their association with HCV and miR-122

The p-body associated protein LSm-1, which has a role in mRNA de-capping promotes HCV replication and translation (139, 141, 156). Interestingly, Lsm-1 has been demonstrated to promote HCV translation by increasing miR-122's effects on translation. Since LSm-1 is not present in miR-122 RISC pull downs, the effect is thought to be independent of Ago2/RISC (156).

As discussed in detail in section 1.2.1.3, the p-body protein Xrn1 inhibits HCV if the genome is not protected by miR-122. However, whether Xrn1 acts on HCV in a p-body is unknown.

1.5 Relationship of p-bodies and p-body proteins with other viruses

Since p-bodies are sites for RNA degradation, it seems rational that RNA viruses would require a mechanism to avoid being targeted to, and degraded in p-bodies. This is particularly important for viruses whose genomes do not resemble mRNA because they have elements such as uncapped 5' ends, or lacking poly A tails, which would normally signal RNA for transport to p-bodies for degradation. Interestingly, some viruses avoid p-body targeting by abolishing their structures in infected cells, while others circumvent normal p-body functions by using them to enhance viral replicative success.

1.5.1 Viruses that disrupt p-body formation

Flaviviruses, West Nile virus (WNV) and Dengue virus infections decrease the presence of visible intracellular p-bodies (2, 157). Inhibition of p-body formation by

these viruses is thought to be advantageous for a few reasons. Firstly, the disruption of p-bodies prevents the virus genome from being degraded by pathways that utilize p-bodies. Secondly, interfering with p-bodies may also promote continuous active cellular translation in the cell, a condition that would be advantageous to WNV and Dengue virus because they require the cell to be actively translating in order to generate viral proteins. Thirdly, disruption of p-bodies could potentially allow WNV and Dengue virus access to p-body proteins (normally sequestered in p-bodies) that the viruses require for efficient replication (158, 159). The mechanism by which p-bodies are inhibited by WNV and Dengue virus is unknown. The disappearance of p-bodies could be the result of the virus directly targeting their assembly, or a consequence of the virus competition for p-body proteins, thus limiting their availability to form visible p-bodies in the cell.

Poliovirus (PV) actively inhibits cellular translation (160). Recently, PV has also been observed to inhibit p-body formation during the mid-phase of its replication cycle (161). PV infections decreased the presence of certain p-body resident proteins, but did not affect the level of any of proteins known to be required for p-body assembly, thus the mechanism by which PV inhibits p-body formation is unclear. P-body disappearance could be the result of PV inhibiting the production of an unknown protein required for p-body assembly, or due to inhibiting expression of a combination of proteins that result in an inability to form p-bodies. The inhibition of p-bodies by PV is speculated to aid PV by preventing its genomic RNAs from being degraded (161).

Influenza-A virus (IAV) nucleoprotein (NP) also interacts with p-bodies and the p-body protein RNA-associated protein 55 (Rap55). Rap55 is required for p-body formation and impedes IAV replication. In addition, as IAV genomic RNA levels increase during infection, p-body abundance decreases. The inverse correlation suggests that p-bodies negatively influence IAV replication, probably by sequestering NP and viral RNA bound by NP within p-bodies. IAV non-structural protein 1 (NS-1) has also been demonstrated to interact with Rap55 later on in IAV infection suggesting that NS-1 prevents p-body assembly and inhibits the recruitment of NP and viral RNA bound by NP to p-bodies (162).

1.5.2 Viruses that utilize p-bodies to augment their replication

Hantavirus transcripts require cellular 5' caps for translation initiation and uses cap snatching to obtain caps from host mRNAs. Interestingly, researchers have demonstrated that hantaviruses obtain 5' caps from host mRNAs inside p-bodies. Hantavirus nucleocapsid (N) protein binds to the 5' cap of cellular mRNA destined for p-bodies. Once in the p-body the mRNA is degraded, but N protein remains bound to the 5' cap preserving it for its own use (163). How N protein facilitates the export of 5' caps from the p-body and, interacts with the Hantavirus proteins and mRNA is unknown.

The Brome Mosaic virus (BMV) has a segmented RNA genome. Two of the RNA segments enter p-bodies and this is thought to be an important step for virus replication (164). BMV has also been demonstrated to require known p-body resident proteins for translation, replication and particle assembly (164, 165).

1.6 The influence of the p-body associated DEAD-box helicase DDX6 on viruses

DDX6, a DEAD-box helicase (Fig. 1.6) is required for p-body formation and is generally considered a translation suppressor (166). Interestingly, this RNA helicase, along with other DEAD-box helicases have also been identified to affect the life cycle of numerous viruses

1.6.1 DEAD-box helicases

DEAD-box helicases are members of the RNA helicase superfamily 2. Superfamily 2 helicases are composed of two RecA-like domains, which contain seven conserved motifs within the helicase core. The name DEAD-box is derived from the conserved amino acid sequence of Asp (D), Glu (E), Ala (A), and Asp (D) found in motif II of the first domain (Fig. 1.6). The N- and C-terminal ends of the protein are thought to define the functionality of different DEAD-box proteins

within the cell. DEAD-box proteins participate in a variety of RNA processing events and can unwind RNA, function as a RNA clamp for recruiting other factors, remove proteins from RNA, act as a chaperone, participate in strand annealing and regulate cellular translation.

As the name suggests DEAD-box helicases can function as helicases. However, they are unique helicases as they do not work processively in a 5' or 3' direction. DEAD-box helicases bind short RNA hairpins and induce strand separation. The helicase domains bind to the backbone of the RNA and thus do not have sequence specificity. The limiting factor of strand separation activity performed by DEAD-box helicases is the length and stability of the hairpin (167), as DEAD-box helicases are ineffective at separating hairpins that are longer than 22 bases (168). Strand separation, regardless of the size of the hairpin, by a DEAD-box helicase requires one bound ATP (169). However ATP hydrolysis is not required to induce a strand separation event, but is required for the disassociation of the DEAD-box helicase from the RNA (170). Determinants in the N- and C- terminal domains of DEAD-box helicases enhance their function. In some DEAD-box helicases, the N- and C-terminal associate with other components, such as proteins and RNA structures, which increase the likelihood of them being loaded onto RNA and initiating a strand separation event (171, 172). Since DEAD-box proteins bind RNA without sequence specificity, the N- and C- terminal ends of DEAD-box proteins can regulate the action of these proteins.

As described above, DEAD-box helicases bind to RNA and after ATP hydrolysis are released from the RNA. However, DEAD-box proteins can also remain clamped on RNA and facilitate the recruitment of other proteins. For example, eIF4AIII is a DEAD-box protein required for the assembly of the exon junction complex (EJC). The EJC is composed of three core proteins, eIF4AIII, MAGOH and Y14. The EJC formation is facilitated by eIF4AIII binding to a hairpin within an mRNA prior to exon ligation. MAGOH/Y14 heterodimers then interact with the RNA bound eIF4AIII, preventing the release of the hydrolysed ATP, thus clamping eIF4AIII to the mRNA, and forming the EJC on the mRNA (173, 174). Removal of the EJC from the mRNA in the cytoplasm could theoretically be mediated by the release

of ADP + Pi from eIF4AIII, which should facilitate the disassociation of eIF4AIII, and the EJC from the RNA, but this has not been demonstrated experimentally.

DEAD-box helicases have also been demonstrated to alter ribonucleoproteins (RNP). Interestingly the *Saccharomyces cerevisiae* DEAD-box helicase Dpb5p has been implicated in mRNA transport from the nucleus to the cytoplasm and is located near nuclear pores. Dpb5p mutants incapable of hydrolysing ATP cause accumulation of mRNA at nuclear pores. The nucleus-restricted mRNAs were further evaluated and observed to contain the nuclear export protein Mex67p. These findings indicate that DpB5p interacts with mRNA and results in the dissociation of Mex67p from the mRNA, allowing the mRNA to enter the cytoplasm (175). There are still no known mechanisms by which DEAD-box proteins displace proteins from the RNA, but evidence suggests that DEAD-box proteins may not require their strand separation ability to accomplish the feat (176). Although there is no suggested mechanism, one can imagine that the process of breaking down RNPs would be regulated tightly, since RNP assembly is probably energy intensive, compared to a small hairpin strand separation event, which only cost the cell 1 ATP molecule.

Similarly to protein function, which requires proper protein folding, RNA also requires proper secondary and tertiary folding for their function. The HCV IRES structured RNA discussed previously exemplifies this. To prevent proteins from taking unfavourable conformations, cells utilize chaperones. For example, as proteins are being translated, the newly added amino acids (aa) can come into contact and make an undesirable interaction that renders the protein inactive. Normally these aas are required for an interaction at a different place within the protein which would allow for the proper folding, however the aa required for the proper interaction has not been added onto the aa chain yet, this allows for an undesired interaction between aas. Chaperones are proteins that temporary interact with a translating protein to either block an undesirable interaction or promote a desirable one. RNAs also require proper secondary and tertiary structure and thus also need to prevent undesirable base pairing and promote correct base pairing. DEAD-box helicases provide a mechanism for the cell to accomplish this task. As

discussed previously, DEAD-box helicases do not recognize a specific sequence and are able to induce strand separation of short RNA duplexes with minimal energy input. These characteristics make DEAD-box proteins ideal candidates as RNA chaperones. The DEAD-box protein CYT-19 has been extensively studied and functions as a RNA chaperone. CYT-19 promotes the correct folding of group I introns of mitochondrial RNA *in-vivo*. Interestingly, there is also evidence that after the correct folding of the intron is achieved, CYT-19 is inhibited from acting on the RNA again. This model of self-regulation exemplifies CYT-19 as an RNA chaperone since a DEAD-box helicase would not be expected to recognize the difference between correctly folded and mis-folded RNA, and would be expected to act randomly on short hairpins (177-179).

In contrast to their helicase activity, DEAD-box proteins also function in strand annealing, and promote the formation of duplex RNA structures. The DEAD-box helicases CrhR and DED1, from cyanobacteria and *Saccharomyces cerevisiae* respectively, are capable of directing strand exchange between two different segments of RNA (180-182). However, to date this phenomenon has only been observed *in-vitro* and has yet to be confirmed in an *in-vivo* system.

DEAD-box helicases are also thought to have the potential to sense metabolites and therefore respond to cellular stress such as glucose shortage. Certain DEAD-box proteins bind AMP. So, when levels are increased during times of cellular stress, binding of AMP by the DEAD-box helicase could act as a form of general cellular regulation leading to up regulation or down regulation of gene expression. The up or down regulation would be the result of AMP-DEAD-box protein increased or decreased affinity for RNA (182, 183).

1.6.2 DDX6 known cellular functions

DDX6 is present at a high concentration in cells and is estimated to out-number the amount of mRNA by seven fold, with the vast majority of the protein associated with p-bodies (184). DDX6 knockdown completely abolishes visible p-body structures and has the most dramatic effect on p-bodies compared to

knockdown of other p-body resident proteins. This is demonstrated by the inability of DDX6 knockdown cells to form p-bodies in the presence of the potent p-body inducer, arsenic, unlike the knockdown of all other p-body resident proteins which still allow p-body formation in the presence of arsenic (166). The core of DDX6 is the DEAD-box helicase, but the protein also contains an N' terminal domain of 61 amino acids which contains glutamine and asparagine that is predicted to interact with other proteins and itself. How DDX6 is able to assemble p-bodies is unknown, but the helicase activity is required (185) and the N' terminal domain is predicted to be required (184).

DDX6 also has a key role in regulating gene expression and maturation of reticulocytes. The final step in the production of mature reticulocytes is the degradation of the mitochondria by the enzyme human reticulocyte 15-lipoxygenase (hr15-LOX) (186). Since reticulocytes do not have a nucleus they are unable to make more mRNA, therefore the mRNA for hr15-LOX must be present in the cell prior to the loss of the nucleus, but not expressed until the proper time in maturation. Regulation of hr15-LOX is mediated by p-body localization. The translation of hr15-LOX mRNA is inhibited by being bound by heterogeneous ribonucleoprotein (hnRNP) K and E1 (187), and DDX6 has been demonstrated to interact with hnRNP K and E1 to localize the proteins and bound hr15-LOX mRNA to p-bodies. Later, and by an unknown mechanism, hr15-LOX is released from p-bodies to induce expression and maturation of reticulocytes (188).

Ernoult-Lange *et al.* have put forth a model to explain how DDX6 suppresses translation based on the observation that DDX6 is present in high concentration in the cell. They suggest that upon suppression, multiple DDX6 proteins are recruited to the mRNA. DDX6 coats the mRNA and causes it to relax its secondary structure. Stalled mRNAs then associate with other stalled mRNAs and assemble a p-body, or alternatively are recruited to a p-body. This model is based on the observations that DDX6 forms multimers in the presence of mRNA (184). DDX6 could then facilitate the degradation of the mRNA, or retain it in storage for re-activation later. DDX6 has also been demonstrated to interact with eIF4E in p-bodies. Although the purpose of this interaction is unknown, it could modulate translation (150).

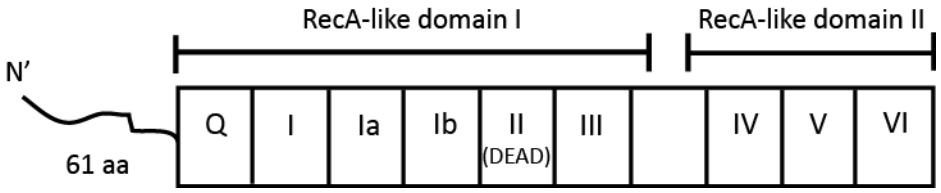

Figure 1.6 The DEAD-box RNA helicase DDX6. A diagram of the DEAD-box RNA helicase DDX6, which is composed of 2 RecA-like domains consisting of 9 motifs. Motif II contains the DEAD amino acid sequence. Motifs Ia, Ib, IV and V are involved in RNA binding while motifs Q, I, II, V and VI contribute to ATP binding. The 61 N-terminal amino acids are predicted to participate in RNA and protein-protein interactions.

DDX6 has also been linked to IRES mediated translation. In neuronal cells under normal cellular conditions, DDX6 interacts with and inhibits translation from the IRES responsible for translating hypoxia inducible factor 1α (HIF-1α). When oxygen is scarce, miR-130a binds to DDX6 mRNA and inhibits its translation. This interaction leads to a decrease in DDX6 proteins, but not DDX6 mRNA and an increase in HIF-1α protein, but not HIF-1α mRNA. These data suggest that DDX6 physically interacts with the HIF-1α IRES to impede HIF-1α mRNA translation (189). A similar observation was made with respect to the vascular endothelial growth factor (VEGF). VEGF promotes blood vessel formation. Its mRNA contains an IRES, and is present at all times in the cell, but is only expressed during times of hypoxia (190). DDX6 has been demonstrated to interact with VEGF IRES and decrease VEGF expression. Conversely, DDX6 knockdown promotes expression of VEGF during times of normoxia suggesting that DDX6 has a role in regulating the VEGF IRES (191). In contrast, DDX6 has been observed to stimulate translation from the c-Myc IRES (192). It is thought that DDX6 up regulates c-Myc by interacting with and unwinding the c-Myc IRES (193, 194).

Interestingly, DDX6 has also been observed to contribute to miRNA gene suppression. siRNA and miRNA silencing in HeLa cells was attenuated when DDX6 expression was blocked. DDX6 also interacts with Ago1 and Ago2, and its role in miRNA and siRNA gene suppression is thought to occur through these interactions. While the contribution of DDX6 to miRNA and siRNA silencing was less than that of Ago2 or GW-182, these observations suggest DDX6 is an active participant in the mechanism of miRNA suppression (153).

1.6.3 The relationship between DDX6 and viruses

There is evidence in the literature that the human immunodeficiency virus (HIV) requires DDX6 for efficient assembly of virus particles. DDX6 knockdown leads to a decrease in HIV particle assembly, and DDX6 is thought to promote assembly through an interaction with HIV gag protein, and potentially HIV genomic RNA (195, 196). However there are also reports suggesting that DDX6 is antiviral

towards HIV, where knockdown of DDX6 led to an increase in HIV replication. The antiviral effects of DDX6 were attributed to DDX6's role in the miRNA suppression pathway, which has been observed to inhibit HIV replication (197-199). Given the conflicting observations reported, the role DDX6 plays in HIV's life cycle is not fully explained and may be quite complex. Similarly, the retrovirus prototype Foamy virus (PFV) utilizes DDX6 for encapsidation of its genome. During virion assembly, DDX6 accumulates at assembly sites and co-localizes with gag, capsid protein, and PFV RNA. The DDX6 helicase domain is required for PFV assembly, and knockdown of DDX6 leads to a decrease in particle release, and the percentage of particles containing genomic RNA (200). The data suggest DDX6 plays an important role in the incorporation of PFV RNA into the capsid.

The adenovirus protein E4 11K interacts with DDX6. E4 11K promotes the accumulation of DDX6 in aggresomes (201), but the implication, purpose and consequence of this interaction, and sequestration of DDX6 into aggresomes is not understood at this time.

As mentioned previously, WNV and Dengue virus affect p-bodies during infection. In the case of WNV many of the p-body proteins including DDX6, LSm1, GW-182 and Xrn1 are recruited to WNV RNA replication sites, and knockdown of several of the recruited p-body proteins leads to a decrease in WNV replication. However DDX6 knockdown was not tested in these experiments and its role in being recruited to WNV replication sites remains unknown (159). Dengue virus 2 (DENV-2) has also been demonstrated to recruit DDX6 to sites of viral replication. Inhibition of DDX6 through siRNA knockdown led to a decrease in DENV-2 assembly and release indicating DDX6 promotes DENV-2 virion production. DDX6 interacts with DENV-2 RNA and can bind two stem-loop structures in the DENV-2's 3'UTR suggesting DDX6 is directly involved in DENV-2 replication (158). Given the ability of DDX6 to interact with DENV-2 RNA, it is probable that DDX6 promotes the DENV-2 life cycle at the RNA replication, translation and/or assembly stage, perhaps to promote translation, or as an RNA chaperone.

DDX6 has also been implicated in the life cycle of HCV. An increase in DDX6 expression was observed in liver biopsies of patients suffering HCV-associated

hepatocellular carcinoma and HCV-related chronic hepatitis (202). Although a direct association of HCV between DDX6 had not been demonstrated, it will be discussed in-length later on.

1.6.3.1 Related DEAD-box helicases and viruses

1.6.3.1.1 DDX1

The cellular function of DDX1 is not well defined, but it localizes to stress granules, sites of mRNA storage, and binds K homology splicing regulatory protein (KSRP) and increases AU-rich element (ARE)-mediated mRNA decay, a degradation pathway based on the recognition of AU elements in the 3'UTR of mRNA (203). DDX1 (and DDX3 as discussed below) also facilitates export of HIV transcripts from the nucleus (204) by promoting oligomerization of Rev protein, a step required for efficient HIV transcript export (205).

DDX1 promotes translation of the double stranded DNA virus JC virus (JCV) (206). It is thought to promote JCV translation by forming a complex with cleavage stimulation factor (CstF), which has been observed to interact with the JCV transcriptional control region (207). Inhibition of DDX1 leads to a decrease in JCV protein expression, supporting DDX1's role in augmenting JCV translation (206).

Utilizing a yeast two-hybrid system, coronavirus exonuclease protein nsp14 was identified to interact with DDX1. Subsequent experiments indicated that DDX1 slightly promoted replication of a coronavirus (208), but the mechanism of replication promotion is unknown. Interestingly, the 3'UTR of the positive genomic strand and the 5'UTR of the negative strand of HCV have also been demonstrated to interact with DDX1 (209). While the purpose of this interaction is unknown, the fact that DDX1 can interact with both the positive and negative strand suggests a role in HCV replication.

1.6.3.1.2 DDX3

DDX3 is a DEAD-box helicase implicated in many aspects of cellular RNA metabolism such as splicing, export, translation regulation (210), and innate immune signalling (211). Not surprisingly due to its ability to stimulate innate immunity, DDX3 affects several viruses including Hepatitis B virus (HBV), HIV, WNV, Japanese Encephalitis virus (JEV) and HCV. DDX3 appears to have an antiviral effect on HBV through interacting with HBV polymerase within the nucleocapsid (212) and by potentially increasing the interferon response in HBV infected cells (213, 214). In the case of HIV, WNV, JEV and HCV, DDX3 is required for efficient viral replication (215).

HIV's dependency on DDX3 for replication has been associated with its role in aiding the transport of HIV transcripts. Unspliced and partially spliced HIV transcripts encode a Rev-response element (RRE), which is bound by HIV Rev protein. Rev is responsible for enhancing the export of HIV transcripts from the nucleus to the cytoplasm. When DDX3 is knocked down, unspliced and partially spliced HIV transcripts bound by Rev, remain in the nucleus. This evidence strongly supports that DDX3 is instrumental in facilitating the transport of Rev bound HIV transcripts (216). The mechanism by which DDX3 facilitates transport of HIV Rev bound transcripts may be similar to the support of mRNA translocation by the DEAD-box helicase Dbp5p of *Saccharomyces cerevisiae*, as discussed previously (216). The HIV Tat protein, which regulates HIV gene transcription, also interacts with DDX3. This interaction leads to an increase in HIV translation and thus promotes HIV success (217, 218).

DDX3 is also required for the HCV life cycle, but its mechanism of action is unclear. Some studies have suggested that the HCV core protein interacts with DDX3 while others suggest a direct role for DDX3 in promoting HCV replication independent from core (219, 220). The role DDX3 plays in WNV has yet to be determined, but similarly to HCV, knocking down DDX3 leads to a decrease in viral replication (159) and DDX3 co-localizes with viral RNA at sites of replication. Similarly, JEV replication is impeded by DDX3 knockdown and is thought to

influence its replication and translation by interacting with the 5' and 3' UTR as well as some of JEV viral proteins (221).

1.6.3.1.3 DDX5

DDX5 (p68) is a co-activator for the expression of several genes and participates in various cell processes. For example, DDX5 enhances Drosha's ability to process certain miRNAs (222, 223) and promotes the splicing of mRNAs (224). Similarly to DDX1, DDX5 was identified using a yeast two-hybrid screen to interact with coronavirus helicase protein nsp13. Knockdown of DDX5 led to a decrease in coronavirus replication demonstrating that DDX5 is proviral, (225) but the mechanism of action is unknown at this time and requires further investigation. Like many of the DEAD-box proteins discussed thus far, DDX5 has also been demonstrated to induce Rev-associated transport of HIV transcripts (226).

During JEV infection, DDX5 is mobilized from the nucleus to the cytoplasm where it interacts with viral proteins NS3 and NS5, and co-localizes with JEV RNA. DDX5 interacts with JEV RNA 3'UTR. Knockdown of DDX5 inhibits JEV replication, but not translation. Therefore, DDX5 interaction with JEV RNA and proteins facilitates JEV RNA replication (227). A role for DDX5 in virion assembly and release is also possible but has not been examined. Similarly to JEV, HCV has also been observed to redistribute DDX5 from the cytoplasm to the nucleus. The redistribution is attributed to an interaction between DDX5 and the C-terminal end of HCV NS5B, and inhibition of DDX5 resulted in a decrease in HCV negative strand synthesis in the context of a full-length replicating construct. However, there was no decrease in negative strand synthesis observed when utilizing a sub-genomic replicon, an RNA in which the structural proteins have been deleted. This suggests that DDX5 may play a role in the switch from RNA replication to virion assembly (228).

1.6.3.1.4 DDX17 and DDX21

Both DDX17 and DDX21 are DEAD-box helicases that promote HIV RNA packaging. DDX17 is required for the alternative splicing of some proteins such as, those involved in the estrogen and androgen signalling pathways (229), while the cellular role of DDX21 is unknown. After siRNA knockdown of both helicases, released HIV particles were less infectious due to a decrease in HIV RNA present in the released particles, indicating that DDX17 and DDX21 aid in the packaging of HIV RNA. There is evidence that both DDX17 and DDX21 interact with HIV Gag protein and this interaction is speculated to be the mechanism by which DDX17 and DDX21 promote viral RNA packaging (230, 231). Since DDX17 and DDX21 perform a similar function it is possible they perform a redundant function or work cooperatively, but this has yet to be examined.

DDX21 has recently been demonstrated to impede IAV RNA replication, by binding to IAV PB1 protein, one of the three viral proteins required to form the IAV RNA polymerase (232, 233). The inhibition of IAV RNA replication by DDX21 is alleviated by IAV NS-1 protein binding to DDX21, which prevents DDX21 from interacting with PB1. DDX21 only effects IAV replication early after infection as NS-1 is produced in higher quantity then PB1, therefore NS-1 outcompetes PB1 for DDX21 binding (232).

1.6.3.1.5 DDX56

DDX56 (NOH61) is a nuclear protein and is suspected to aid in the assembly of the 60S ribosomal subunit (234). It is not required for WNV RNA replication but associates with WNV capsid protein and influences the production of infectious particles (235). Through mutational analysis, the C-terminal end of DDX56 was found to be responsible for the interaction with the capsid protein. Knockdown of DDX56 leads to a decrease in infectious particles generated within the cell, but not in the efficiency by which particles are released, indicating that DDX56-capsid interaction positively influences the incorporation of viral genomes into particles. In

order for successful incorporation of WNV genomes, DDX56 helicase activity is required, thus suggesting that DDX56 helps to remodel the WNV genome into a form capable of being incorporated into a particle (236).

1.6.3.1.6 Modulators of intracellular immunity

As there are numerous DEAD-box proteins in the cell, which do not generally bind with specificity, many have been linked to cellular signalling. As such many DEAD-box proteins interact with antiviral pathways within the cell. Therefore, many DEAD-box proteins have the potential to be antiviral or proviral, depending on how the protein influences their activity. Many of these scenarios have been discussed in depth and reviewed by Fullam *et al*. and will not be discussed in length here, but is an important concept to retain when considering DEAD-box proteins and viruses (211).

1.7 HCV treatment strategies

1.7.1 Current therapy

As mentioned briefly at the beginning of this chapter, the classical treatment for HCV is a combination therapy composed of ribavirin and pegylated-IFNα. This treatment was administered for 48 weeks in patients with genotype 1, 4, 5 and 6, and for 24 weeks in patients with genotype 2 and 3 (237). Unfortunately, this treatment was accompanied by severe side effects such nausea, malaise, anemia, and depression, which led many people to quit the therapy early. In addition, the treatment was not always effective, in particular, the treatment only cleared the virus in 40-50% of the patients with genotype 1 and upwards of 80% in the other genotypes (238). Clearance of the virus is determined as less than 50 UI/ml of HCV in the patients' blood 24 weeks post treatment and is deemed as a sustained virological response (SVR). If after 24 weeks of treatment, the HCV RNA is higher the 50 UI/ml, the patient is deemed a non-responder (238).

Given the lack of effectiveness of pegylated-IFNα and ribavirin against genotype 1, viral protease inhibitors were recently added to the combination therapy, known as triple therapy. Boceprevir or telaprevir were both recently approved for HCV treatment and act to inhibit genotype 1 NS3/4A protease (9). Regardless of the protease inhibitor used, the triple therapy has a duration of 48 weeks. However, the timing and inclusion of the protease inhibitor varies depending on the inhibitor selected, the patients previous treatment, and ongoing response to the treatment (239). Through triple therapy, viral clearance for genotype 1 has increased to 70-80%. Unfortunately, boceprevir and telaprevir only inhibit genotype 1. Similar to the pegylated-IFNα and ribavirin treatment, triple therapy also has severe side effects for the patient (9, 239).

Very recently, two new DAAs have been approved for use in Canada. Sofosbuvir is a nucleoside analogue and can be used in combination with pegylated-IFNα and ribavirin to treat genotype 1 and 4, or with ribavirin alone to treat genotype 2 and 3 (240). Simeprevir is another NS3/4A protease inhibitor and is approved for use in combination with pegylated-IFNα and ribavirin (241).

1.7.2 Potential future treatments

The current treatments, which include DAA have provided an increase in efficacy, but still are not 100% effective at clearing HCV, and are accompanied with adverse side effects for the patients due to the inclusion of pegylated-IFNα. Therefore, there is still much room for improvement to the standard of care, and the future goal is the development of an effective combination therapy that excludes pegylated-IFNα.

1.7.2.1 Future DAA and host manipulation treatment

The recent introduction of new effective DAAs against HCV is just the first wave of new DAAs. Many other DAAs in phase II and III clinical trials display fewer side effects and can achieve SVR without the inclusion of pegylated-IFNα and

ribavirin. The exclusion of pegylated-IFNα and ribavirin has led to a dramatic decrease in the side effects. These DAA include nucleoside analogs, non-nucleoside inhibitors, and inhibitors against NS3/NS4A, and NS5A. It appears that the future of HCV therapy will remain a combination therapy, but the combination will only be DAAs targeting various aspects of HCV (242).

1.7.2.2 Targeting host factors to inhibit HCV

There are two treatments that have entered phase II clinical trials that inhibit host factors that result in a decrease in HCV replication. One of the treatments is based on the observation that compounds that interfere with cyclophilin A inhibit HCV (9). Cycolphilin A is a cellular chaperone protein that is thought to interact with NS5A and modify its confirmation in a pro-viral fashion (73). The second treatment is based on HCV's dependency for miR-122. Utilizing a 15-nucleotide locked nucleic-acid modified antisense oligonucleotide that binds with high affinity to the 5'end of miR-122 (LNA-122), miR-122 can be sequestered. The sequestration removes miR-122 and potently inhibits HCV replication. In clinical trails, LNA-122 is highly effective at reducing HCV levels in humans and Chimpanzee while not inducing escape mutants or causing adverse side effects (103, 243).

1.7.2.3 siRNA targeted degradation

Interestingly, small double stranded silencing RNA (siRNA) potently inhibit HCV replication in cell culture (244-246). This type of treatment takes advantage of the fact HCV is a positive sense RNA virus, and thus is readily targeted for siRNA mediated degradation. siRNAs have been designed that target conserved sequences within the HCV genome. The theory is that the siRNA will recognize the virus and cleave the genome, and if the siRNA targets a conserved (and essential) sequence, the virus will be unable to escape the siRNA by incorporation of point mutations to the target sequence. Although preliminary data has indicated that HCV specific siRNAs are highly effective, none have progressed to clinical trials. This could be

due to the fact, even though the siRNA target conserved regions, HCV still has demonstrated enough flexibility in those regions to mutate and escape the siRNA (247, 248).

1.8 Summary

This literature review has discussed the current knowledge regarding HCV and its life cycle, with an emphasis on HCV's unique relationship with the miRNA, miR-122. Much has been discovered regarding the association of HCV, miR-122 and components of the miRNA suppression pathway. However the role of p-bodies, and the proteins that comprise p-bodies have yet to be fully elucidated. Thus, a portion of this literature review not only discussed what is known about p-bodies and HCV, but what p-bodies are, and their known role in the life cycle of other viruses. This literature review also focused on the core p-body protein DDX6 and other DEAD-box RNA helicases. The focus on DDX6 is based on its elevation in the liver of patients with HCV associated HCC, with no known function in supporting HCV life cycle. Given that DDX6 is a DEAD-box helicase, its reason for being elevated in HCV associated HCC could be associated with an unknown cellular function of DDX6 and/or other observed and predicted functions of DEAD-box helicases within the cell and with other viruses. Lastly, this literature review focuses on the past and current therapy for patients infected with HCV and the great strides made towards very effective treatment with an emphasis on the encouraging results of targeting miR-122-HCV relationship as a possible treatment.

2.0 HYPOTHESES AND OBJECTIVES

2.1 Rationale

HCV has an intricate and complicated dependency on miR-122. This dependency includes many components of the miRNA suppression pathway including the Ago proteins. DDX6 has been demonstrated to interact with Ago1 and Ago2 and is required for miRNA suppression of mRNA and p-body formation and maintenance. DDX6 is also up regulated in HCV associated HCC and HCV-related chronic hepatitis suggesting that the helicase may be involved directly or indirectly in the HCV life cycle and pathogenesis. These observations suggested to us that DDX6 might affect the HCV life cycle through a role in the mechanism of action of miR-122. However, the fact that other flaviviruses such as WNV and Dengue also disrupt p-bodies and recruit DDX6 to sites of viral replication, suggest it is possible that HCV might also utilize DDX6 in a non-miR-122 dependent manner as well.

To enhance HCV replication, miR-122 binds through Watson-Crick base pairing to two miR-122 binding sites within the HCV 5'UTR. Because HCV requires miR-122 binding for efficient replication, the miR-122 binding region is highly conserved in all HCV genotypes. The conservation of nucleotides and the requirement of miR-122 to bind in conjunction with RISC components suggests that this area should also be accessible to siRNA/RISC complexes, and should be a prime target for antiviral inhibition by inducing siRNA mediated degradation. In addition, although the miR-122 binding region is highly conserved, constant pressure placed on the virus by repeated siRNA treatment will reveal the tolerance for mutations in the miR-122 binding region.

2.2 Hypotheses

i. HCV requires the DEAD-box helicase DDX6 for effective replication.

ii. DDX6 affects HCV replication by supporting miR-122 augmentation of HCV replication.

iii. Binding of the miR-122/Ago2 complex to the conserved miR-122 binding region in HCV 5'UTR is required for efficient HCV replication, which makes it a target for siRNA cleavage as a therapeutic strategy.

iv. If the sequences targeted by miR-122 binding site targeting siRNAs are not essential for HCV replication, then after several rounds of treatment with siRNA targeting the miR-122 binding region, viruses having mutations within the miR-122 binding sites will be selected and their analysis will reveal the tolerance for point mutations in this region.

2.3 Objectives

i. Determine the effect of DDX6 depletion on HCV translation and RNA amplification.

ii. Determine if the effects of DDX6 depletion on HCV translation and replication are dependent or independent of the effects of miR-122 on HCV translation and replication.

iii. Design siRNA that target the miR-122 binding region and assess their ability to inhibit HCV replication.

iv. Assess the ability of miR-122 binding site targeting siRNAs to inhibit an HCV genome containing a point mutation within the target region.

v. Treat Huh7.5 cells repeatedly with siRNAs targeting the miR-122 binding region to assess the evolution of virus resistance to the siRNAs.

vi. Sequence the 5'UTR of the viral RNAs selected in objective v. to identify the specific escape mutants that have evolved.

3.0 MODULATION OF HEPATITIS C VIRUS RNA ACCUMULATION AND TRANSLATION BY DDX6 AND miR-122 ARE MEDIATED BY SEPARATE MECHANISMS

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Keywords: Hepatitis C virus; DDX6; microRNA; miR-122; replication; translation; processing body.

Running title: DDX6 does not regulate HCV via miR-122

Published in PLOS ONE June 24, 2013

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3.2 Authors' contribution

Adam Huys performed all the experiments described and reported in the manuscript with the exception of Figure 7. Patricia A. Thibault performed some of the experimental replicates used in Figure 7. Adam Huys wrote the manuscript, which was edited by Patricia A. Thibault and Joyce A. Wilson.

3.3 Abstract

DDX6 and other p-body proteins are required for efficient replication of Hepatitis C virus (HCV) through unknown mechanisms. DDX6 has been implicated in miRNA induced gene silencing, and since efficient HCV replication and translation relies on the cellular microRNA, miR-122, we hypothesized that DDX6 had a role in the mechanism of action of miR-122. However, by using multiple HCV translation and replication assays, we have found this is not the case. DDX6 silencing decreased HCV replication and translation, but did not affect the ability of miR-122 to stimulate HCV translation or promote HCV RNA accumulation. In addition, the negative effect of DDX6 silencing on HCV replication and translation was not dependent on miR-122 association with the HCV genome. Thus, DDX6 does not have a role in the activity of miR-122, and it appears that DDX6 and miR-122 modulate HCV through distinct pathways. This effect was seen in both Huh7.5 cells and in Hep3B cells, indicating that the effects are not cell type specific. Since infections by other viruses in the *Flaviviridae* family, including Dengue and West Nile virus, also disrupt p-bodies and are regulated by DDX6, we speculate that DDX6 may have a common function that support the replication of several Flaviviruses.

3.4 Introduction

Processing bodies (p-bodies) are transient cellular compartments where mRNAs are degraded and sometimes stored (97, 146, 249). P-bodies are composed of an array of proteins such as mRNA de-capping, de-adenylating, and RNA exoribonuclease enzymes, many of which have been implicated in miRNA suppression and mRNA turn-over. The composition, location, and number of p-bodies in a cell is dynamic and based on mRNA degradation requirements (146). Fewer p-bodies are found in cells under conditions of increased mRNA translation, due to a reduced need for mRNA degradation, and conversely, when mRNA degradation is promoted by impeded cellular translation, p-bodies are found in greater numbers and increased size.

microRNA-mediated mRNA silencing involves both the suppression of translation, and induction of mRNA degradation (250). The process of miRNA silencing involves binding of miRNA and associating proteins Argonaute (Ago) and GW-182. GW-182 then associates with Poly(A)-binding protein (PABP) and several host protein components of the deadenylation complexes, which is believed to be at least part of the mechanism by which miRNAs suppress translation and promote mRNA degradation (251, 252). P-bodies are the likely sites of miRNA-induced mRNA degradation since they contain high concentrations of miRNAs, Ago and GW-182 (143). The resident p-body protein DDX6 (RCK, p54) is essential for the assembly and maintenance of p-bodies, and depletion of DDX6 inhibits p-body formation, even when stimulated by arsenite, a robust translation inhibitor and p-body inducer (166). DDX6 and its homologues from different species such as *Saccharomyces cerevisiae* (Dhh1), *Xenopus laevis* (Xp54) and *Caenorhabditis elegans* (CGH-1) are members of the DEAD-box RNA helicase family and bind to RNA with high affinity. Once bound, DDX6 has the ability to modify the secondary structure of the RNA in an energy independent and dependent manner (184, 185, 188). In addition, the *S. cerevisiae* DDX6 homologue, Dhh1, stimulates decapping of mRNAs by decreasing the rate of translation, presumably by exposing the cap to decapping

enzymes (253, 254). DDX6 is also believed to enhance miRNA gene suppression, as its knockdown leads to an alleviation of miRNA suppression in HeLa cells (153).

Some RNA viruses have been shown to disrupt p-bodies during infection, while others appear to use them as sites for replication, assembly, and release; thus, the relationship between p-bodies and RNA viruses has been the focus of extensive study (157, 164, 200, 255, 256). Of particular interest is the Hepatitis C virus (HCV) a human pathogen that causes liver cirrhosis, liver failure and hepatocellular carcinoma. HCV, a 9.6kb positive strand RNA virus and member of the *Flaviviradae* family, has been demonstrated to alter p-body distribution during infections (257). The role of the redistribution of p-bodies during HCV infection is unknown, but p-bodies themselves do not appear to be required for HCV replication (257, 258). However, knocking down p-body proteins Lsm-1, PatL-1, Ge-1, GW-182, Ago2, and DDX6 results in reduced HCV replication, indicating a direct or indirect role for these proteins in supporting the HCV life cycle (111, 139, 141, 259). DDX6 protein levels are also frequently elevated in HCV-associated carcinomas, while being down-regulated in other liver carcinomas, suggesting a possible role in HCV-induced liver pathology (202). Knocking down DDX6 in cell culture reduces HCV RNA replication, but there is conflicting evidence regarding whether DDX6 silencing decreases HCV translation (141, 259). Scheller *et al.* (141) found that DDX6 knockdown reduced HCV translation levels while Jangra *et al.* (259) observed no effect on translation. DDX6 has been demonstrated to co-precipitate with HCV core protein, and through binding to core, associate with HCV RNA (259). DDX6 also localizes near HCV replication centers, suggesting it may play a role in trafficking or regulating HCV RNA (139, 257, 259). Thus the function of DDX6 in HCV replication needs further study in order to better understand the relationship and its possible link to hepatocellular carcinoma.

HCV requires miR-122, an abundantly expressed liver-specific miRNA, to efficiently establish an infection (105), however the mechanism of action of miR-122 is unknown. The relationship between miR-122 and HCV is unusual in that unlike conventional miRNA-mRNA interactions, which normally take place between the miRNA seed region (the 5’ nucleotides 2-8) and sequences in the 3’ UTR of mRNA,

miR-122 binds to two tandem seed binding sequences within the HCV 5' UTR (104, 105). In addition, instead of down-regulating translation and RNA stability, miR-122 promotes viral RNA accumulation, mostly by stabilizing the HCV genome, although it can also stimulate translation (108, 110, 114, 260); and a direct role for miR-122 in promoting viral genome replication has not been ruled out (113). Like in miRNA suppression, annealing between the seed sequences of miR-122 to the HCV genome is required for activity, but unusually, so too are some of the nucleotides outside of the seed sequence; in particular, nucleotides 15 and 16 at the miR-122 3' end anneal to sequences at the 5' end of the HCV genome, creating an RNA overhang which likely protects the uncapped HCV 5' terminus from access by RNA degradation enzymes (106). Lastly, the space between the two miR-122 binding sites, and Ago2 are also crucial for miR-122 augmentation of HCV RNA accumulation (104, 111, 140). Importantly, using miR-122 antagonists to block the activity of miR-122 in both chimpanzees and humans dramatically decreased serum HCV titres, making miR-122 a promising target for antiviral treatment and highlights the importance of miR-122 and the miRNA pathway in HCV life cycle (103). As a result, efforts to understand the mechanism of action of miR-122 are ongoing.

Because DDX6 knockdown attenuates miRNA suppression activity, we hypothesized that DDX6 may modulate HCV replication and translation by mediating the activity of miR-122, or *vice versa*. Jangra *et al.* showed that DDX6 knockdown did not affect the ability of miR-122 to augment HCV replication (259), and our goal was to expand on these studies by using several model HCV replication and translation assay systems to confirm whether there is, or is not, a connection between the influence of DDX6 and miR-122 on the HCV life cycle. First, our observations confirm that DDX6 knockdown modulates both HCV translation and replication. Next we show that the DDX6 is not required for miR-122 to affect translation, nor is miR-122 annealing required for DDX6 to affect HCV translation. In addition, by using various assays to analyze HCV RNA accumulation, including a novel assay in which HCV replicates independently from miR-122 (116), we have confirmed both that DDX6 is not required for the activity of miR-122 on HCV RNA accumulation, and that miR-122 is not required for the influence of DDX6 on HCV

replication. These data are strong indicators that, although both DDX6 and miRNAs are located within p-bodies and are implicated in miRNA suppression activity, they do not affect HCV replication and translation through a common mechanistic pathway.

3.5 Materials and methods

3.5.1 Cell culture

Huh7.5 cells (261) were used for all experiments unless otherwise stated, and were grown in D-MEM supplemented with 10% fetal bovine serum, 0.1ηM non-essential amino acids (Wisent, Montreal, Canada), and 100 units/ml Pen/Strep (Life Technologies, Burlington ON, Canada). Hep3B cells (ATCC number HB-8064) are a human hepatoma cell line and were grown under the same conditions as Huh7.5 cells.

3.5.2 Plasmids and DNA probes

The pSGR JFH-1 Fluc WT sub-genomic replicon was provided by Dr. T. Wakita (262) and the full-length genome constructs pJ6/JFH-1 (p7-Rluc2A), pJ6/JFH-1 (p7-Rluc2A) GNN, (herein called J6/JFH-1 Rluc and J6/JFH-1 Rluc GNN) were provided by Dr. Charles M. Rice (263). pJ6/JFH-1 Rluc p34, pJ6/JFH-1 Rluc p34 GNN, and pSGR JFH-1 p3 were described previously (111, 116). The plasmids pT7Luc and pRL-TK were obtained from Promega Co. (Madison, WI). pLuc-122x2 and pLuc-122x2 S1+S2:p3-4 were kindly provided by Dr. Peter Sarnow (104), and the plasmid pRL-TK CXCR4 4x was provided by Dr. Tariq M. Rana (153).

3.5.3 Small interfering RNAs (siRNA), duplex microRNA (miRNA), and miR-122 antagonist sequences

All small RNAs were synthesized by Thermo-scientific Dharmacon Inc (Lafayette, CO). The target sequence for the siRNA used for siControl was GAGAGUCAGUCAGCUAAUCA and siDDX6 was ACCCGAGGUAUUGAUAUACAA. The sequence for the duplex miRNA were as follows: miControl, GAGAGUCAGUCAGCUAAUCA; miCXCR4 antisense, UGUUAGCUGGAGUGAAAACUU; miCXCR4 sense, GUUUUCACUCCAGCUAACACA; miR-122, UGGAGUGUGACAAUGGUGUUUGU; miR-122p3 UGCAGUGUGACAAUGGUGUUUGU; miR-122p3-4 UGCUGUGUGACAAUGGUGUUUGU; miR-122*, AAACGCCAUUAUCACACUAAAUA. miR-122, miR-122p3, and miR-122p34 duplex were formed by annealing the indicated miRNA guide strand with miR-122*. The miR-122 antagonist, hsa-miR-122a miRIDIAN microRNA Hairpin Inhibitor, bears a proprietary sequence.

3.5.4 *In-vitro* RNA transcription

HCV RNA transcripts were prepared from *Xba*I-linearized plasmids as described previously (111) by using the MEGAScript T7 High Yield Transcription Kit, while pRL-TK was linearized with *Bgl*II to generate Rluc mRNA and pT7 luciferase was linearized with *Xmn*I to generate Fluc mRNA using mMessage mMachine T7 Transcription Kit (Life Technologies**,** Burlington, ON, Canada) (116).

3.5.5 Electroporation of Huh7.5 and Hep3B cells

Huh7.5 and Hep3B cells were electroporated as previously described (116).

3.5.6 Transient HCV replication assays

Huh7.5 cells were initially electroporated with 60pmol of siRNA to achieve knockdown of the desired protein. Three days post-electroporation, cells were electroporated again with 1µg of HCV RNA, 1ug of control mRNA, 60pmol of siRNA, and 60 pmol of miRNA, if applicable. Electroporated cells were re-suspended in 8mL of media and plated for luciferase assays, protein analysis, RNA analysis, and cell number assays. Cells where harvested 3 days post-electropoartion unless otherwise specified. An additional luciferase assay sample was harvested at 1-hour post-electroporation to confirm electroporation efficiency. Experiments with Hep3B cells were conducted using the same method, except that 5µg of HCV RNA was used instead of 1µg.

3.5.7 Transient HCV translation assays

Huh7.5 cells were electroporated on day 0 with 60pmol of siRNA in order to silence the gene of interest. The cells were electroporated again on day 3 with 5ug of J6/JFH-1 RLuc GNN or J6/JFH-1 RLuc p34 GNN RNA and 1ug of Fluc capped mRNA. Immediately prior to the second electroporation a sample of the cells was harvested for analysis of protein knockdown by western blot. After the second electroporation, cells were plated for luciferase assays, RNA analysis and cell number assays and were harvested 3.5 hours post-second-electroporation.

3.5.8 Translation suppression assays

Huh7.5 cells were electroporated with 60pmol of siRNA on day 0, resuspended in 8ml of media and then 300µl was seeded into each well of a 24-well plate. Two days post-electroporation the cells were transfected with 100ng of plasmids pLuc-122x2 or pLuc-122x2 S1+S2:p3-4, and pRL-TK; or pRL-TK CXCR4 4x and pLuc-122x2 S1+S2:p3-4 (104) and 0.05 – 5pmol of miRNA per well using

Lipofectamine 2000 (Life Technologies, Burlington, ON, Canada) and the suggested protocol. Cells were harvested 24 hours post-transfection for luciferase assays.

3.5.9 Luciferase assays

Luciferase assays were performed as previously described (111). Briefly, the cells where washed with phosphate-buffered saline (PBS), and lysed into 100µl of the appropriate lysis buffer. Luciferase levels were assayed by using Renilla Luciferase, Firefly Luciferase, or Dual Luciferase assay kits (Promega Co., Madison. WI, USA) and light emission was measured by the Glomax 20/20 Luminometer (Promega Co., Madison, WI, USA).

3.5.10 Cell number assay

Cell numbers were calculated three days post-electroporation or 24 hours post-transfection using WST-1 reagent. The WST-1 assay was performed according to the protocol provided by Roche (Roche Canada, Mississauga, ON, Canada). Cell numbers were determined by comparing them to a standard curve.

3.5.11 RNA purification

Cells were harvested into 1mL of Trizol (Life Technologies, Burlington, ON, Canada) and RNA purified using the manufacturer's recommended protocol.

3.5.12 Northern blot analysis

Northern blots were conducted as previously described (111).

3.5.13 Real-time PCR analysis of RNA

RNA was reverse-transcribed using the iScript reverse transcription kit (Bio-Rad Inc., Missassauga, ON, Canada). DDX6 mRNA (Life Technologies, Hs00898913) and GAPDH mRNA (used as internal control [#4352934E]) levels were quantified using TaqMan (Life Technologies) probes, primers, and protocol. HCV and Fluc RNA levels where determined using primers directed towards the Renilla (RLuc) gene in the reporter HCV genomic RNA, and Fluc in the control mRNA as described previously (112)

3.5.14 SDS-Page and western blot analysis

Protein samples were collected by lysing equal number of cells in SDS-PAGE protein lysis buffer (10% SDS, 5% beta-mercaptoethanol, 20% glycerol, 0.2M Tris-HCl, pH 6.8, 0.05% bromophenol blue). Samples were electrophoresed through 10% SDS-polyacrylamide gels and transferred onto a Hybond-C Extra nitrocellulose membrane (GE Healthcare, Mississauga, ON, Canada). Blots were probed with primary antibodies (1:5000) mouse monoclonal anti-actin (AC-15; Abcam, Cambridge, MA, USA) and (1:5000) rabbit anti-RCK (DDX6 antibody; Bethyl labs, Montgomery, TX, USA). Blots were then probed with (1:1000) anti-mouse (800nm) and anti-rabbit (680nm) infrared dye-linked secondary antibody (Li-Cor Biosciences, Lincoln, NE) and visualized using a Li-Cor Odyssey infrared imager and knockdown was determined using Odyssey Infrared Imaging System Application Software Version 3.0.

3.5.15 Fluorescence microscopy

Huh7.5 cells were plated onto an 8-chamber slide. The cells were fixed using paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were then exposed to primary antibody, human IC6 polyclonal antibody (264) (a gift from

Marvin Fritzler), followed by secondary antibody, Alexa fluor®594 Goat anti-human IgG (H+L) (Life Technologies, Burlington, ON, Canada). Fluorescence images were obtained using a Zeiss axiovert 200M inverted microscope at a magnification of 63 x 10 and the Axiovision 4.6 software.

3.5.16 Statistical analyses

Data are presented as the average of least three independent experiments, unless otherwise indicated, and error bars represent the standard deviation (SD). Data analysis was carried out with Prism 5 software. P values, unless otherwise indicated were calculated by using Student *t*-test *P<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.6 Results

3.6.1 Depletion of DDX6 reduces p-body abundance

Following DDX6-specific or control siRNA treatment, levels of DDX6 protein, mRNA and p-bodies were evaluated by western blot, qRT-PCR, and microscopic analysis. Huh7.5 cells treated with DDX6 siRNA expressed 79% ± 9% less DDX6 protein and 81% less mRNA, compared to cells treated with control siRNA (Fig. 3.1A and B). DDX6 knockdown also significantly reduced the abundance of visible p-bodies (Fig. 3.1D). P-body disruption was enumerated by assessing the number of p-bodies per cell in random fields of 100 cells. Only 9% of the Huh7.5 cells treated with siDDX6 contained two or more p-bodies in contrast to 96% in cells that had been treated with control siRNA (Fig. 3.1C).

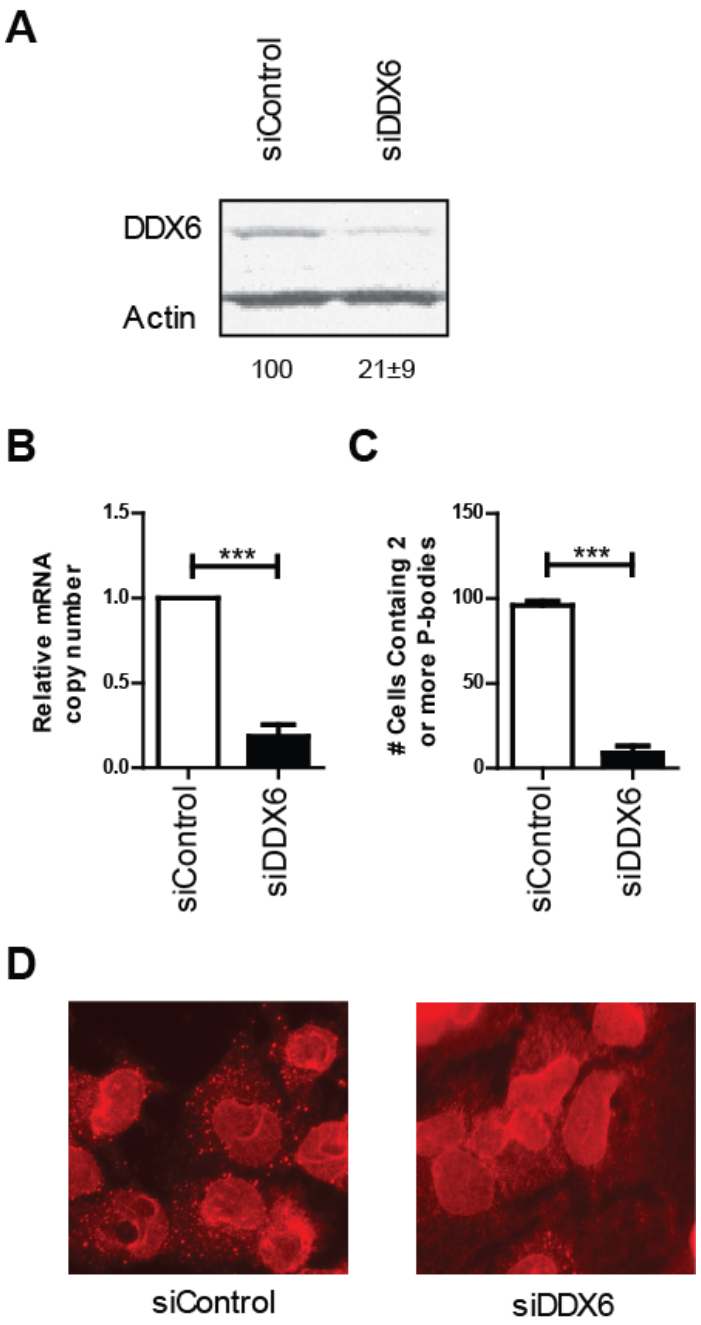

Figure 3.1 DDX6 specific siRNA, siDDX6, depletes cells of DDX6 and disrupts p-body formation. (A) Western blot analysis shows siDDX6 protein levels in siControl and siDDX6 treated cells. The values represent the average relative DDX6 protein levels and standard deviation from western blot analyses of 12 independent experiments (B) qRT-PCR analysis show that siDDX6 depletes cells of DDX6 mRNA (C) Enumeration of cells containing two or fewer p-bodies after siRNA treatment. (D) Immunoflorescence staining of p-bodies by staining for the p-body protein (Ge-1) in DDX6 depleted cells and control cells.

3.6.2 Silencing of DDX6 attenuates replication of both full-length and sub-genomic HCV replicon RNA

Three days following DDX6 knockdown, replication of both bi-cistronic JFH-1 replicon RNA (SGR JFH-1 FLuc) and full-length J6/JFH-1 RLuc HCV RNA was evaluated. Both RNAs contain luciferase reporter genes so that HCV replication could be evaluated based on luciferase expression. At 3 days post-electroporation luciferase expression from sub-genomic and full-length HCV replicons was decreased by 30% and 45% respectively in DDX6 silenced cells (Fig. 3.2A and B). Reduced HCV replication was not attributed to a defect in cell proliferation, since WST-1 assays indicated that DDX6 silencing did not significantly affect cell numbers present at the time of harvest (Fig. 3.2C and D), and DDX6 knockdown was confirmed by western blot (data not shown). These results are similar to, and confirm, those reported by Scheller *et al.,* Jangra *et al.,* and Pager *et al.* (139, 141, 259).

3.6.3 DDX6 knockdown suppresses HCV translation

The effect of DDX6 on HCV translation was examined by co-electroporating DDX6-depleted and control cells with non-replicating full-length HCV RNA carrying a Renilla luciferase reporter gene (J6/JFH-1 Rluc GNN) and capped firefly luciferase mRNA (FLuc). Relative HCV translation levels were determined by calculating the ratio of Renilla luciferase *vs.* firefly luciferase expression. Knockdown of DDX6 reduced HCV translation of full-length J6/JFH-1 Rluc GNN by 46% compared to cells treated with control siRNA (Fig. 3.3A, miControl). The phenotype attributed to DDX6 silencing is consistent with that previously reported by *Scheller et al*. (141). However, we saw inconsistent data regarding whether DDX6 knockdown affected HCV translation. Results obtained using the identical method in an earlier passage of Huh7.5 cells indicated that DDX6 silencing did not affect HCV translation (Fig. 3.4). This data is consistent with those reported by Jangra *et al.* (259). Quantitative RT-

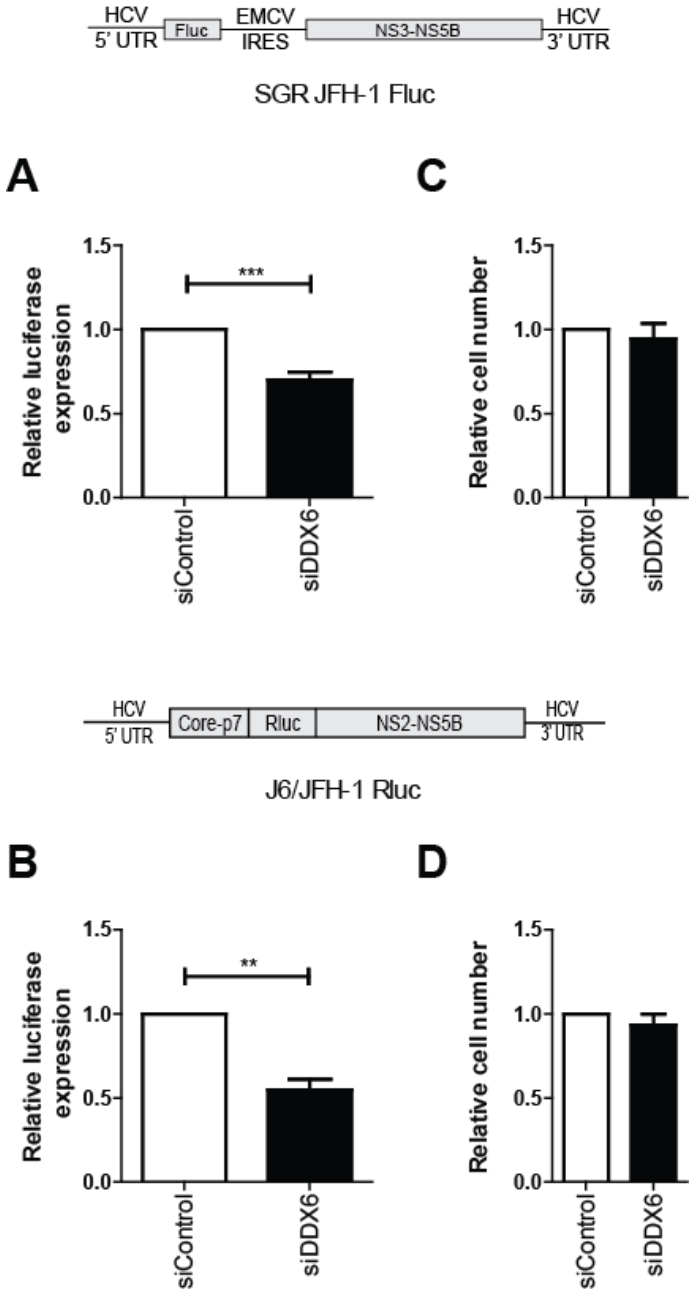

Figure 3.2 DDX6 depletion attenuates sub-genomic and full-length HCV replication. Relative luciferase expression levels in cells electroporated with (A) sub-genomic JFH-1 Fluc replicon RNA (SGR JFH-1 FLuc), or (B) full-length J6/JFH-1 Rluc RNA. (C) Relative cell numbers from A three days after electroporation. (D) Relative cell numbers from B three days after electroporation.

Figure 3.3

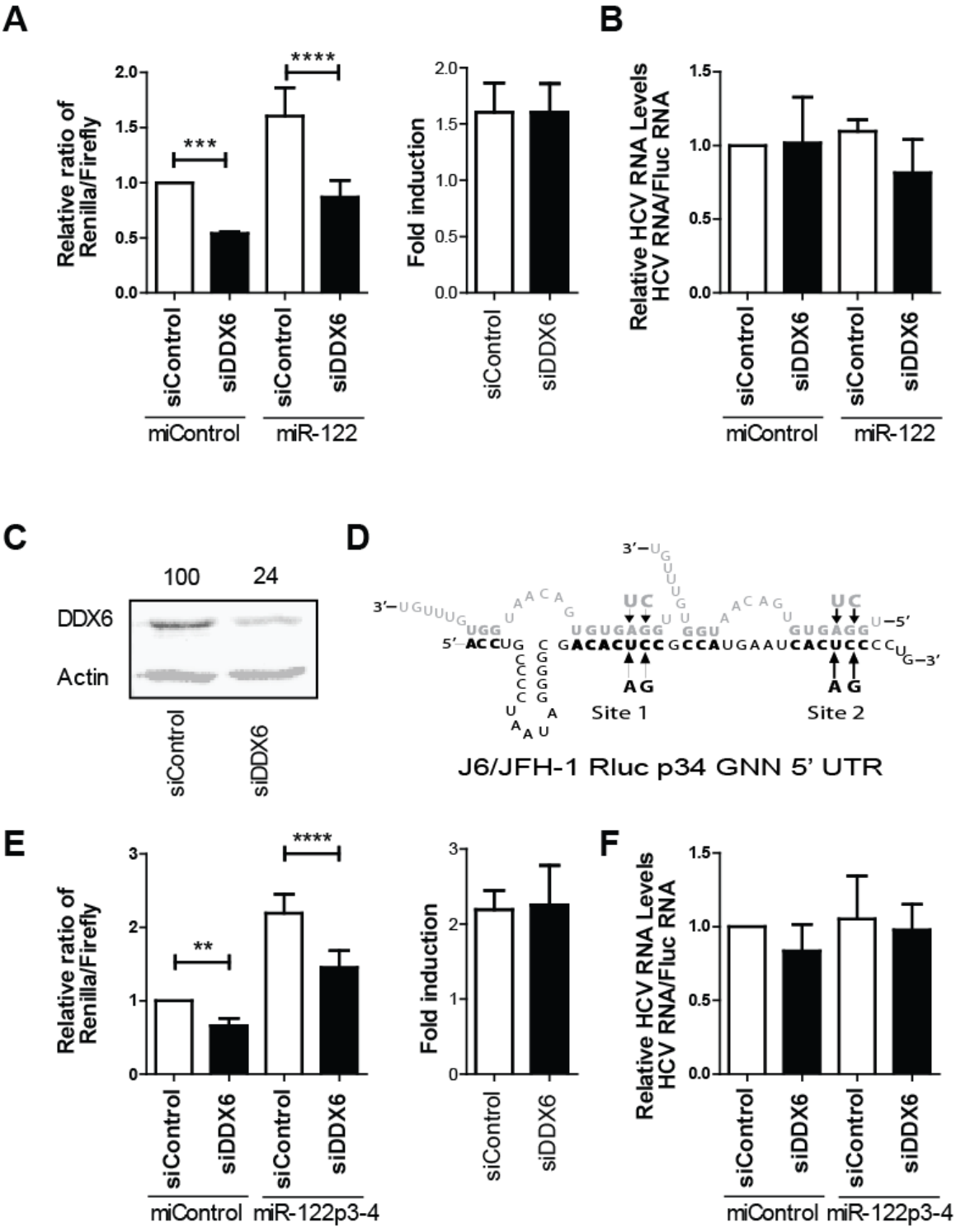
A
Relative ratio of Renilla/Firefly
2.0
1.5
1.0
0.5
0.0
***
****
siControl
siDDX6
siControl
siDDX6
miControl
miR-122
Fold induction
2.0
1.5
1.0
0.5
0.0
siControl
siDDX6
B
Relative HCV RNA Levels HCV RNA/Fluc RNA
1.5
1.0
0.5
0.0
siControl
siDDX6
siControl
siDDX6
miControl
miR-122
C
100
24
DDX6
Actin
siControl
siDDX6
D
3′–UGUUUG
5′–ACCUG
UGG
UGUGAGG
GGU
GACACUCCGCCAUGAAUCACUCCCCUG–3′
AG
AG
Site 1
Site 2
J6/JFH-1 Rluc p34 GNN 5′ UTR
E
Relative ratio of Renilla/Firefly
3
2
1
0
**
****
siControl
siDDX6
siControl
siDDX6
miControl
miR-122p3-4
Fold induction
3
2
1
0
siControl
siDDX6
F
Relative HCV RNA Levels HCV RNA/Fluc RNA
1.5
1.0
0.5
0.0
siControl
siDDX6
siControl
siDDX6
miControl
miR-122p3-4

Figure 3.3 siDDX6 depletion decreases HCV translation, but does not affect miR-122 stimulation of HCV translation. (A) Relative Rluc:Fluc expression in Huh7.5 cells co-electroporated with full-length, replication defective (J6/JFH-1 Rluc GNN) HCV RNA containing a Rluc reporter, a capped Fluc mRNA, and the indicated siRNA and miRNA. The graph on the right shows the relative fold translation stimulation by miR-122. (B) Relative RNA ratios of J6/JFH-1 Rluc GNN to capped firefly mRNA measured by qRT-PCR. (C) Western blot analysis show that siDDX6 depletes cells of DDX6 protein compared to siControl. (D) A schematic drawing of the 5' UTR of J6/JFH-1 Rluc GNN RNA showing WT and mutant miR-122 binding sites (J6/JFH-1 Rluc GNN p34) and annealing pattern with the corresponding miRNA miR-122, or miR-122p34. (E) Relative Rluc:Fluc expression from J6/JFH-1 Rluc GNN p34 RNA co-transfected with capped Fluc mRNA, and the indicated siRNA and miRNA . The graph on the right shows the relative fold translation stimulation by miR-122p34. (F) Relative RNA ratios of J6/JFH-1 Rluc GNN p34 to capped firefly mRNA measured by qRT-PCR. Data in (A) represents the average of 5 independent experiments and the data in (E) represents the average of 8 independent experiments. Significance was determined by performing a one-way ANOVA with Bonferonni's multiple comparison test.

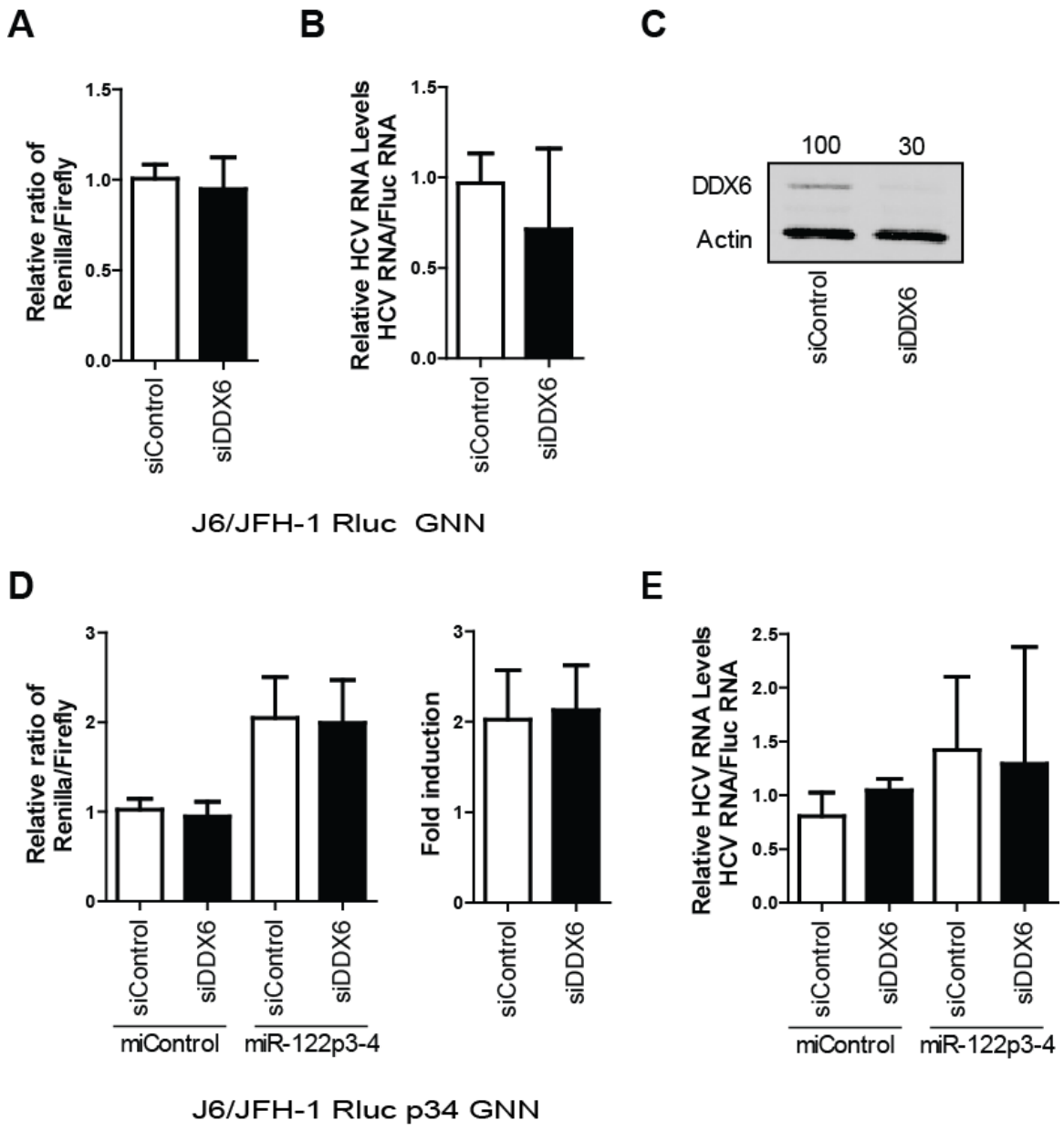

Figure 3.4 In a subset of experiments we observed that HCV translation was not inhibited by DDX6 knockdown. (A) Relative luciferase expression of J6/JFH-1 Rluc after electroporation with siDDX6 or siControl. (B) Relative RNA ratios of J6/JFH-1 Rluc GNN to capped firefly mRNA measured by qRT-PCR. (C) Western blot analysis of cell lysates confirming knockdown of DDX6. (D) Relative luciferase expression of J6/JFH-1 Rluc m34 in presence and absence of miR-122 p34. The graph on the right shows the relative fold translation stimulation by miR-122p34. (E) Relative RNA ratios of J6/JFH-1 Rluc GNN p34 to capped firefly mRNA measured by qRT-PCR.

PCR quantification of HCV and control Fluc RNA support that the influence of DDX6 and miR-122 on HCV were on translation rather than RNA stabilization (Fig. 3.3B and Fig. 3.4B). A western blot confirmed efficient DDX6 knockdown in the cells treated with siDDX6 (Fig. 3.3C, Fig. 3.4C).

3.6.4 DDX6 knockdown does not affect the efficiency of miR-122 stimulation of HCV translation

Since we hypothesized that DDX6 was involved in the activity of miR-122, we expected DDX6 knockdown to attenuate the ability of miR-122 to stimulate HCV translation. To test this we analyzed HCV translation stimulation by miR-122 following knockdown of DDX6. Co-electroporation of miR-122 with J6/JFH-1 Rluc GNN stimulated HCV translation 1.6 fold, and siRNA depletion of DDX6 did not affect the efficiency by which miR-122 stimulated translation (Figure. 3.3A, miR-122). Thus, DDX6 silencing had no affect on the ability of miR-122 to stimulate HCV translation. To further confirm our observations, we assayed the effect of DDX6 knockdown on HCV translation stimulation by an exogenously provided synthetic miR-122 (miR-122p34). In these experiments, cells were electroporated with J6/JFH-1 Rluc p34 GNN, which contains two point mutations in the miR-122 binding sites, and therefore can no longer bind endogenous miR-122, but can associate with a synthetic miR-122 containing compensatory mutations (Fig. 3.3D). Knockdown of DDX6 attenuated translation of J6/JFH-1 Rluc p34 GNN by 34%, indicating that miR-122 binding to the HCV genome is not required for the influence of DDX6 on HCV translation (Fig. 3.3E, miControl). In addition, miR-122p34 stimulated HCV translation by approximately 2 fold in both DDX6 depleted and control cells (Fig. 3.3E, and 3.4D, miR-122p34). Quantitative RT-PCR data suggest that the observed effects on translation of J6/JFH-1 Rluc p34 GNN were the result of a decrease in translation and not a decrease in genome stability (Fig. 3.3F and 3.4E). From these results we confirmed that the effects of DDX6 and miR-122 on HCV translation are functioning independently.

3.6.5 DDX6 knockdown does not affect miR-122 augmentation of HCV replication

Supplementing Huh7.5 cells with miR-122 increases HCV RNA accumulation in infected cells (104, 110-112). Since we hypothesize that the role of DDX6 in HCV replication is linked to the activity of miR-122, we expect that the ability of miR-122 to augment HCV replication will be attenuated by DDX6 knockdown. To assess this question we examined the efficiency of miR-122-mediated augmentation of HCV replication with and without DDX6 knockdown by assessing replication-competent HCV RNA accumulation by northern blot and replication by luciferase reporter expression. Northern blot analysis (Fig. 3.5A and B) confirmed that DDX6 silencing reduced RNA accumulation by 45% in miControl cells, and by 50% in miR-122-treated cells, further supporting the role of DDX6 in enhancing HCV RNA accumulation. When the cells were supplemented with miR-122, J6/JFH-1 RNA levels increased (Fig. 3.5A and B), confirming that miR-122 supplementation augments HCV accumulation. Importantly, the ability of miR-122 to augment HCV replication was not affected by DDX6 knockdown and miR-122 supplementation increased J6/JFH-1 RNA abundance by about 2.0-fold in siControl and siDDX6-treated cells (Fig. 3.5C). These observations were confirmed by analysis of luciferase expression where miR-122 supplementation caused a 2.3 fold increase in luciferase expression in both control and DDX6 knockdown cells (Fig. 3.5D and E). Knockdown of DDX6 did not affect cell growth (Fig. 3.5F), nor modify translation of a co-electroporated capped Fluc mRNA significantly (Fig. 3.5G), and DDX6 knockdown was confirmed by western blot analysis (Fig. 3.5H). These results indicate that DDX6 is not required for miR-122 augmentation of HCV replication.

Figure 3.5

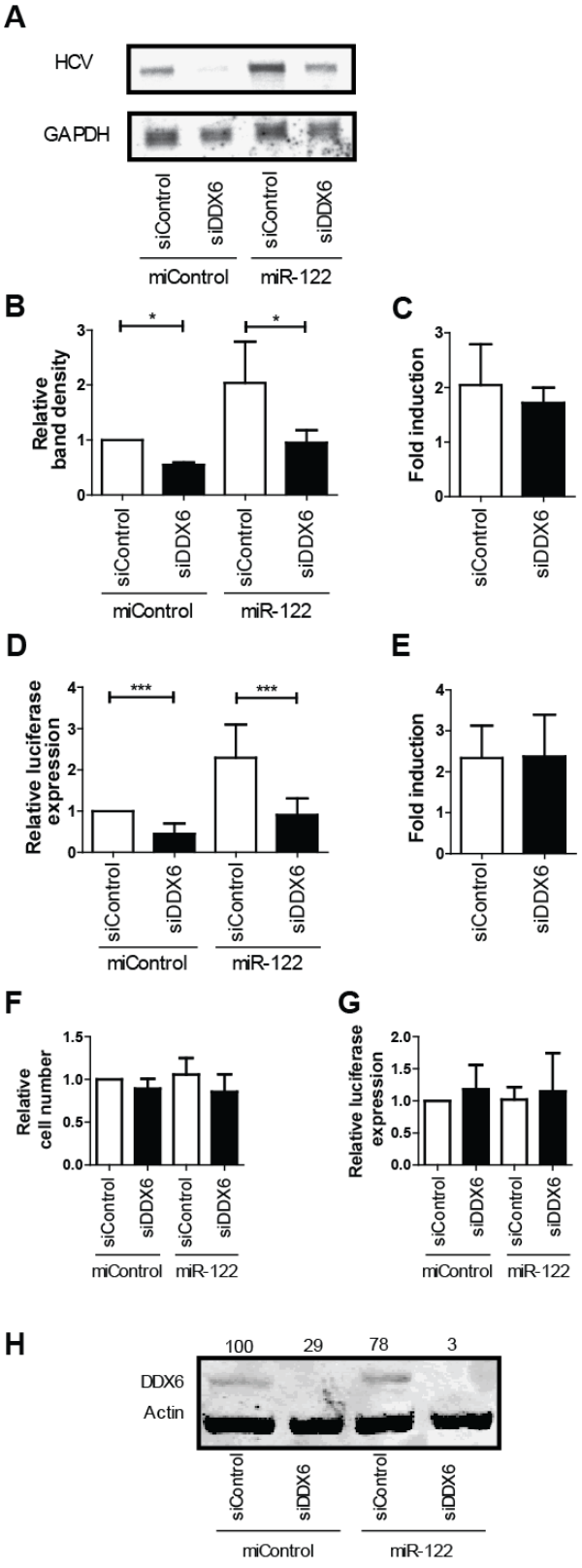
A
HCV
GAPDH
siControl
siDDX6
siControl
siDDX6
miControl
miR-122
B
Relative band density
C
Fold induction
D
Relative luciferase expression
E
Fold induction
F
Relative cell number
G
Relative luciferase expression
H
100
29
78
3
DDX6
Actin

Figure 3.5 Augmentation of HCV replication by miR-122 is not dependent on DDX6. (A) Representative northern blot analysis of HCV and GAPDH RNA from cells 3 days after electroporation with J6/JFH-1 Rluc RNA and the indicated siRNA and miRNA. (B) Average band intensity measured from 3 independent northern blots as shown in part A, and (C) the corresponding fold induction of RNA accumulation by miR-122. (D) Relative luciferase expression 3 days post-electroporation in the same experiments as shown in parts A, B and C, and (E) the fold induction of luciferase expression by miR-122. (F) Relative cell numbers and (G) Fluc expression from a co-electroporated capped mRNA at 2 hour post electroporation. (H) Western blot analysis of DDX6 protein expression. Data in (D-G) represents the average of 8 independent experiments. A one-way ANOVA with Bonferonni's multiple comparison test was performed on F and G to show they where not significantly different.

3.6.6 DDX6 silencing impedes both miR-122-independent and miR-122-dependent HCV replication in Huh7.5 cells

We had established that DDX6 is not required for the effects of miR-122 on HCV replication and next wished to determine if the opposite was also true; that miR-122 is not required for the influence of DDX6 on HCV replication. Our lab has recently reported that sub-genomic HCV JFH-1 replicon RNA (SGR-JFH-1) was capable of replicating in Hep3B and Huh7.5 cells without the influence of miR-122 (116). miR-122-independent HCV replication was confirmed by using replicon RNAs having a single point mutation in both miR-122 binding sites (SGR JFH-1 S1, S2:p3) that renders the replicon unable to bind endogenous miR-122 (Fig. 3.6A). This system provides a means to screen for genes having a role in the activity of miR-122, since their knockdown should affect miR-122-dependent HCV replication but not miR-122-independent HCV replication. In proof-of-principle experiments, depletion of Ago2, a protein having a role in the activity of miR-122, affected miR-122-dependent but not miR-122-independent HCV replication (116). We used this system to test whether the effects of DDX6 depletion on HCV replication were dependent on or independent from miR-122 activity. Data indicated that DDX6 functions independently from miR-122 since DDX6 knockdown reduced SGR JFH-1 S1, S2:p3 (miR-122-independent) replication at all three time points analysed (Fig. 3.6B), and relative to control knockdown cells was 59%, 65%, and 54% lower at days 1, 2, and 3 respectively (Fig. 3.6C). DDX6 knockdown reduced replication of SGR JFH-1 WT (miR-122-dependent) by 80%, 61%, and 67% on days 1, 2, and 3 respectively (Fig. 3.6C). The relative decrease of miR-122-independent *vs.* miR-122-dependent HCV replication induced by DDX6 knockdown was not statistically significantly different on days 2 and 3 (Fig. 3.6C). This data indicates that the effect of DDX6 on HCV replication is not dependent on the activity of miR-122.

Figure 3.6

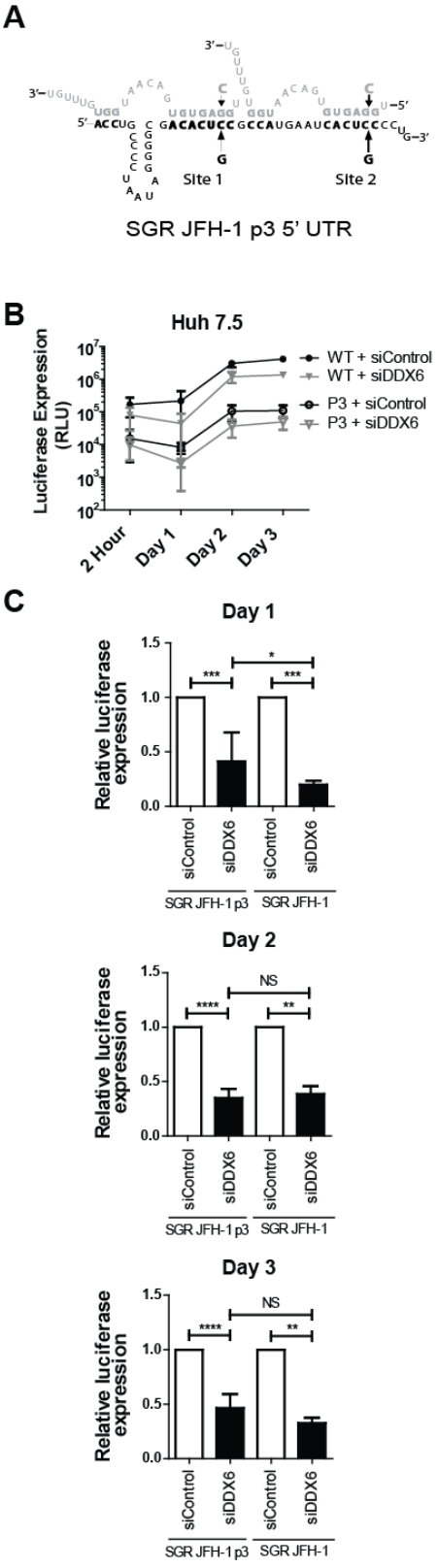
A
Site 1
Site 2
SGR JFH-1 p3 5' UTR
B
Huh 7.5
Luciferase Expression (RLU)
WT + siControl
WT + siDDX6
P3 + siControl
P3 + siDDX6
2 Hour
Day 1
Day 2
Day 3
C
Day 1
Day 2
Day 3
Relative luciferase expression
siControl
siDDX6
SGR JFH-1 p3
SGR JFH-1
NS

Figure 3.6 Both miR-122-dependent and miR-122-independent HCV SGR RNA replication in Huh7.5 cells is attenuated by depletion of DDX6. (A) A schematic drawing of the 5' UTR of the JFH-1 sub-genomic replicon (SGR JFH-1) with a single point mutation in both miR-122 binding sites (SGR JFH-1 p3) that abolishes endogenous miR-122 binding, and the corresponding miRNA containing compensatory mutations to reinstate binding (miR-122p3). (B) Time course of luciferase expression in Huh7.5 cells electroporated with WT SGR JFH-1 (miR-122-dependent replication) and SGR JFH-1 p3 (miR-122-independent replication) treated with either siDDX6 or control siRNA. (C) Luciferase expression, relative to siControl, on day 1, 2, and 3 post-electroporation for the samples shown in panel B.

3.6.7 DDX6 silencing impedes both miR-122-independent and miR-122-dependent HCV replication in Hep3B cells

We also confirmed the phenotype of DDX6 knockdown on miR-122-independent and miR-122-dependent HCV replication in Hep3B cells. DDX6 knockdown decreased miR-122-independent replication in Hep3B (SGR JFH-1 S1, S2:p3 + miControl) by 32%, 56%, and 45% on days 1, 2, and 3 respectively (Fig. 3.7A and B) and was not significantly different from the decrease in replication observed in cells supporting miR-122-dependent HCV replication (SGR JFH-1 p3 + miR-122p3), which were 49%, 78%, and 54%. This data confirms that DDX6 depletion affects HCV replication independent of miR-122, and demonstrates that the influence of DDX6 on HCV replication is not specific to Huh7.5 cells.

3.6.8 miRNA translation suppression is slightly attenuated by DDX6 knockdown

Others have reported that siRNA knockdown of DDX6 leads to a decrease in the ability of miRNA to silence their targeted genes (153). To confirm an effect of DDX6 on miRNA suppression activity in Huh7.5 cells, we tested silencing activity of endogenous miR-122, exogenous miR-122p34, and exogenous miCXCR4 in a plasmid-based miRNA suppression assay, with and without knockdown of DDX6 (Fig 3.8). For the assays, cells were co-transfected with plasmids encoding luciferase (Fluc or Rluc) bearing miR-122, miR-122p34 or miCXCR4 miRNA target sites in the 3'UTR (Fig. 3.8A, B, and C; top row) and a control plasmid expressing the opposite luciferase, either Fluc or Rluc, to control for transfection efficiency (Fig. 3.8A, B, and C; top row). Knockdown of DDX6 increased reporter expression by 35% compared to control cells (Fig. 3.8A) indicating that DDX6 silencing attenuated suppression by endogenous miR-122. In a positive control, co-transfection of a miR-122 antagonist increased luciferase expression by 350% and confirmed that endogenous miR-122 suppressed translation of the reporters (Fig. 3.8A, αmiR-122). To confirm that the

Figure 3.7

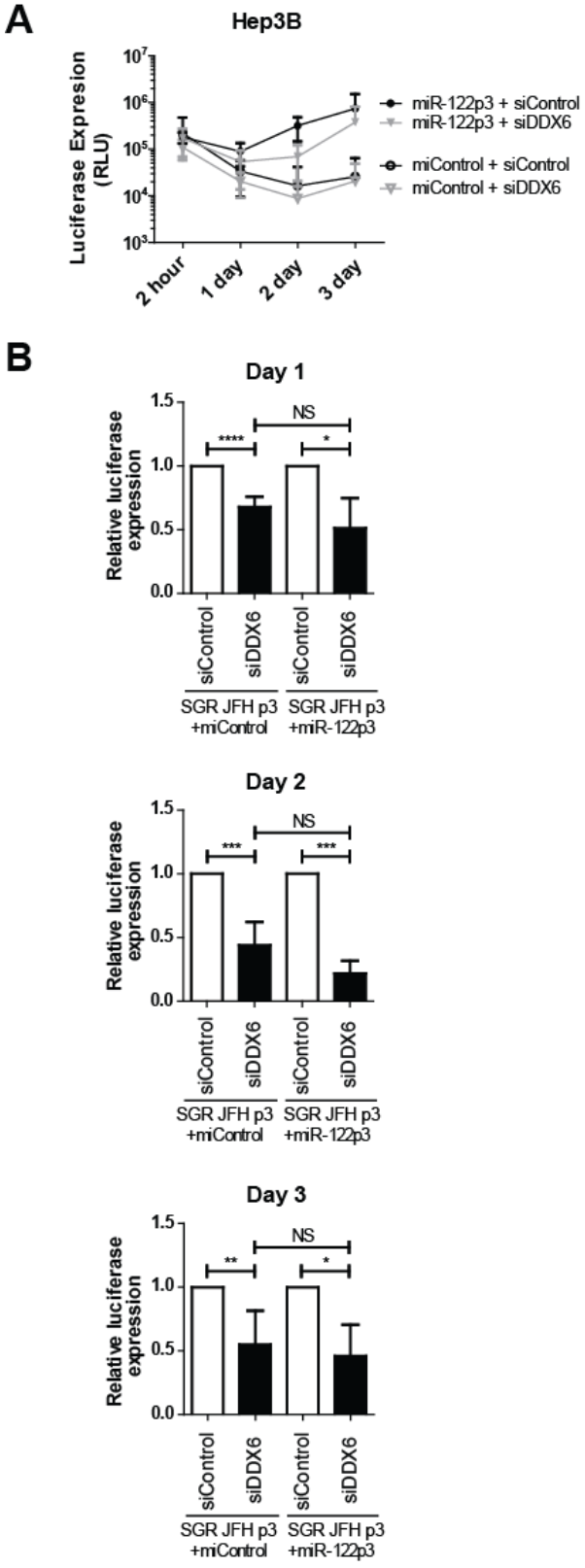
A
Hep3B
Luciferase Expresion (RLU)
10^7
10^6
10^5
10^4
10^3
2 hour
1 day
2 day
3 day
miR-122p3 + siControl
miR-122p3 + siDDX6
miControl + siControl
miControl + siDDX6
B
Day 1
Relative luciferase expression
1.5
1.0
0.5
0.0
****
NS
*
siControl
siDDX6
siControl
siDDX6
SGR JFH p3 +miControl
SGR JFH p3 +miR-122p3
Day 2
Relative luciferase expression
1.5
1.0
0.5
0.0
***
NS
***
siControl
siDDX6
siControl
siDDX6
SGR JFH p3 +miControl
SGR JFH p3 +miR-122p3
Day 3
Relative luciferase expression
1.5
1.0
0.5
0.0
**
NS
*
siControl
siDDX6
siControl
siDDX6
SGR JFH p3 +miControl
SGR JFH p3 +miR-122p3

Figure 3.7 Both miR-122-dependent and miR-122-independent HCV SGR p3 RNA replication in Hep3B cells is attenuated by depletion of DDX6. (A) Time course analysis of luciferase expression from SGR JFH-1 p3 RNA in Hep3Bs cells co-electroporated with either miR-122p3 (miR-122-dependent) or miControl (miR-122-independent) and the indicated siRNAs. (B) Luciferase expression from SGR JFH-1 p3 RNA, relative to siControl, in the presence or absence of miR-122 p3 at days 1, 2, and 3.

effects of DDX6 knockdown on miRNA suppression was not due to a decrease in miR-122 biogenesis, we also tested DDX6 knockdown using a system in which gene suppression was induced by serial dilutions of an exogenously provided synthetic miR-122p34 (Fig. 3.8B). In this assay, increased amounts of transfected miR-122p34 caused greater suppression of luciferase expression, and DDX6 knockdown attenuated suppression by miR-122p34 by 11% and 8% when 0.5 and 0.125pmol of miR-122p34 was used, but not significantly with other dilutions (0.25 and 0.06pmol) miR-122p34 (Fig. 3.8B). Although DDX6 knockdown showed statistically significant alleviation of miR-122 silencing, the effect was modest and the physiological relevance questionable. To confirm that our observations were not specific to suppression by miR-122, we also analysed the effects of DDX6 knockdown on miRNA suppression by another miRNA, miCXCR4, using a reporter plasmid containing miCXCR4 binding sites. This assay is identical to one used previously to identify a link between miRNA suppression activity and DDX6 (153). We observed statistically significant alleviation of miRNA suppression by miCXCR4 following DDX6 knockdown (Fig. 3.8C), but the effects were relatively small, and not as robust as those previously reported (153), which suggests that DDX6 is not essential for miRNA suppression activity in Huh7.5 cells.

3.7 Discussion

It has been known for several years that both DDX6 and miR-122 support HCV replication. miR-122 functions to support HCV replication by using at least some of the miRNA pathway proteins, with Ago2 having a key role (111, 112, 114). Since DDX6 interacts with Ago proteins, and both are abundant in p-bodies, we hypothesized that the functions of DDX6 and miR-122 would be linked. However, in spite of the close association of Ago2 and DDX6, and their shared implication in the mechanism of miRNA silencing, extensive evidence indicates that the primary mechanisms by which DDX6 regulates HCV translation and replication is not related to the role of miR-122. Specifically, DDX6 is not required for the effects of miR-122

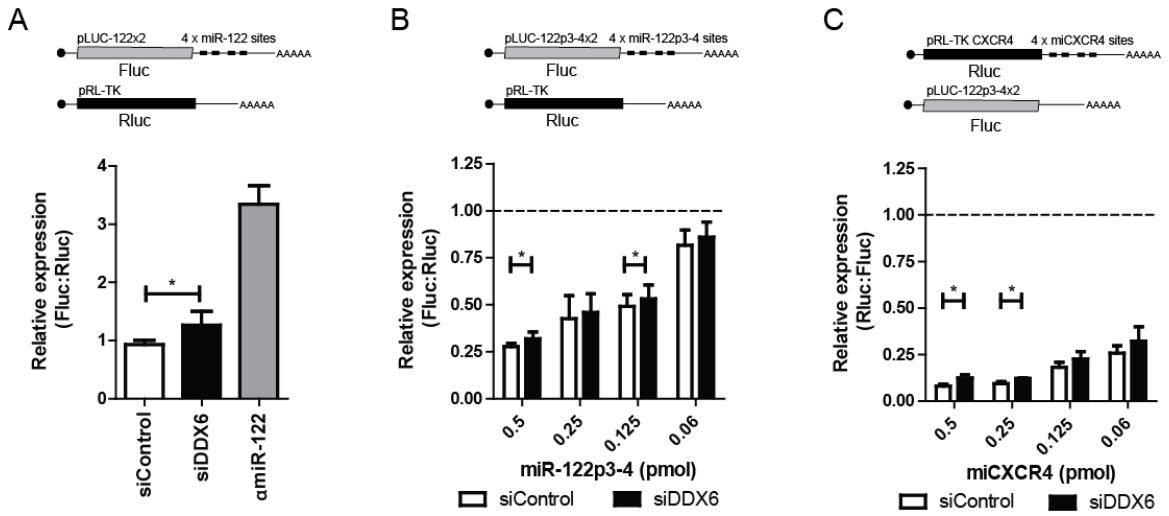

Figure 3.8 miRNA translation suppression by endogenous and exogenous miRNA is alleviated by DDX6 silencing. (A) Schematic diagram of the mRNAs expressed from the co-transfected reporter plasmids used in this miRNA suppression assay. Expressed mRNAs carry the Fluc sequence with 4 WT miR-122 binding sites in the 3' UTR, or a control Rluc sequence. Relative Fluc:Rluc expression from the reporters was assessed in control, DDX6-depleted, and miR-122 antagonist-treated Huh7.5 cells. (B) Schematic diagram of the mRNAs expressed from the co-transfected reporter plasmids used in this miRNA suppression assay. Expressed mRNAs carry the Fluc reporter gene and 4 mutant miR-122p34 binding sites in the 3' UTR, or a control Rluc gene. Relative Fluc:Rluc expression from the reporters was assessed in control or DDX6-depleted cells, co-transfected with the indicated amounts of miR-122p34. (C) Schematic diagram of the mRNAs expressed from the co-transfected reporter plasmids used in this miRNA suppression assay. Expressed mRNAs contain an Rluc gene with 4 miCXCR4 miRNA binding sites in the 3' UTR or a control Fluc gene. Relative Rluc:Fluc expression from the reporters was assessed in control or DDX6-depleted cells that were co-transfected with the indicated amounts of miCXCR4. Data in A represents the average of 6 experiments and B represents the average of 4 experiments.

on HCV replication, and miR-122 association with the HCV genome is not required for the effects of DDX6 on HCV translation and replication. Our data support those of Jangra *et al*, who reported that DDX6 was not required for miR-122 augmentation of HCV replication (259), and we further confirm that DDX6 is also dispensable for miR-122 stimulation of HCV translation. In addition, we confirm that miR-122 is not required for DDX6 to influence HCV replication by showing that DDX6 knockdown still attenuates HCV replication in our miR-122-independent HCV replication assays. However, because there is a general trend showing that DDX6 knockdown affects miR-122-dependent replication slightly more strongly than miR-122-independent replication, and at one time point the difference was statistically significant (Fig. 3.7C, 1 day), we cannot exclude the possibility that a second function, requiring both miR-122 and DDX6, has a minor role in supporting HCV replication.

Our studies support the findings of Scheller *et al*., that DDX6 regulates HCV translation (141), however, in experiments performed in a different passage of Huh7.5 cells, DDX6 silencing had no effect on HCV translation, and our data were similar to those reported by Jangra *et al.* (259). We cannot explain why HCV translation in different passages of Huh7.5 cells has different requirements for DDX6, but variation in the phenotype and the efficiency by which Huh7-derived cells support HCV replication during Huh7.5 cell passage has been well documented (265, 266). Regardless of whether DDX6 knockdown does or does not affect HCV translation, in both cases miR-122 was equally capable of stimulating HCV translation in control and DDX6 depleted cells, and thus all of our translation data supports the conclusion that DDX6 is dispensable for miR-122 stimulation of HCV translation.

In addition, our data does not indicate that DDX6 plays a major role in the mechanism of miRNA suppression, which was previously observed in HeLa cells (153). DDX6 knockdown resulted in a statistically significant attenuation of miRNA gene silencing activity; however, the physiological significance of a role for DDX6 in miRNA suppression activity in Huh7.5 cells is questionable since DDX6 only attenuated suppression by a small amount. Nonetheless, we cannot rule out the possibility that other proteins present in Huh7.5 cells have redundant functions in

mediating miRNA suppression, which may explain the discrepancy between our results and those reported in HeLa cells.

The mechanisms of action of DDX6 and miR-122 in supporting HCV replication remain unclear. miR-122 is believed to modulate the efficiency of HCV RNA accumulation by stabilizing genomic RNA (106, 113). This is likely mediated by miR-122 masking and thus protecting the uncapped 5' end of the viral genome from degradation by Xrn1, another p-body protein, but a direct role for miR-122 in the process of HCV replication has also been suggested (106, 113). In addition, the influence of Xrn1 knockdown on HCV replication is variable. In some cases, siRNA knockdown of Xrn1 has been reported to increase HCV replication (113) and in other cases it was reported to have no effect or to decrease HCV replication (139, 141, 258). These data suggest that perhaps p-body proteins have multiple functions in up-regulating and down-regulating HCV replication.

HCV infections alter p-body structure and recruit p-body proteins such as DDX6, Lsm-1, Pat-1, Xrn1, and Ago2, to lipid droplets and sites of HCV replication (139, 159, 257, 258). Gene knockdown studies indicate roles for several of these proteins in supporting HCV RNA accumulation and recent evidence indicates that re-localization of p-body proteins during virus infections is not unique to HCV. The yeast DDX6 homolog, Dhh1 is required to recruit Brome mosaic virus genomic RNA to sites of replication in a yeast model replication system (267). In addition, other members of the *Flaviviridae* family including Dengue and West Nile virus also disrupt p-body structure and recruit p-body proteins to replication sites, to positively regulate virus replication (158, 159). Thus, DDX6 and other p-body proteins may have a common role in supporting virus life cycles (158, 159). That other Flaviviruses utilize DDX6 to support their life cycles, but are not modulated by miR-122 (or by other miRNAs that we know of), also supports the notion that the role of DDX6 (and perhaps other p-body proteins) is not linked to the activity of miR-122. However, we cannot omit the possibility that re-localization of p-body proteins may support Flavivirus replication by using mechanisms that overlap those of miR-122.

Biochemical characterization of DDX6 reveals a possible function in the life cycle of viruses. DDX6 binds to mRNA without sequence specificity, and relaxes its secondary structure (184). This activity requires ATP binding but not ATP hydrolysis in a way resembling RNA chaperones that stabilize RNA (184). In a model proposed by Ernoult-Lange *et al*., DDX6 binds to an mRNA, first as part of a translation repression complex, and then as individual proteins that coat translation-stalled mRNA, and unfolds it in preparation for degradation in p-bodies (184). DDX6 association with Dengue virus stem-loops, the DB1 and DB2 structures, in the 3' UTR, is required for efficient virus replication (158). We speculate that DDX6 could associate with and unfold virus genomes in preparation for initiation of genome replication, however thus far DDX6 has only been reported to associate with HCV genomes through association with the HCV core protein.

DDX6 has also been implicated in the efficiency of HCV virion release and in the assembly of HIV virions (139, 200). The possible role of DDX6 in HCV virion assembly must be separate from its activity in promoting replication since its knockdown attenuates replication of sub-genomic HCV replicons, which do not express core nor assemble particles. However, the association of DDX6 with HCV core protein, and with lipid droplets, could perhaps suggest a role in remodelling viral genome in preparation for virion assembly.

3.8 Acknowledgements

We are grateful to Takaji Wakita, Charles Rice, Tariq Rana, and Marvin Fritzler for constructs and reagents. A.H acknowledges the University of Saskatchewan College of Graduate Studies, and the Department of Microbiology and Immunology for funding. P.A.T would also like to acknowledge the NSERC and the National CIHR Research Training Program in Hepatitis C virus (NCRTP-HepC) for masters and doctoral scholarships. J.A.W acknowledges funding provided by the University of Saskatchewan, National Science and Engineering Research Foundation (RGPIN-342475) and Saskatchewan Health Research Foundation, (RAPID 1927).

4.0 CONCURRENT ADVANCES IN UNDERSTANDING THE RELATIONSHIP BETWEEN DDX6, P-BODIES, miR-122 AND HCV

At the time our research was being conducted, there were numerous other research groups evaluating the relationship between HCV, p-body structures, proteins associated with p-bodies, and the miRNA suppression pathway. Many, but not all of the findings of the other groups supported our on-going observations, and a common model for the roles of p-bodies in the HCV life cycle emerged.

4.1 DDX6 supports HCV replication but not through miR-122

Concurrent with our work, five papers where published that reported DDX6 knockdown decreases HCV replication (139, 141, 257-259). All of these reports confirmed that DDX6 silencing led to a decrease in HCV RNA accumulation (139, 141, 257-259). We also confirmed by using several different methods that DDX6 augments HCV replication in a manner that is independent from miR-122 and support the findings of Jangra *et al*. (259). Thus, we conclude that DDX6 is not part of the pathway in which miR-122 affects HCV replication, and that p-bodies and possibly p-body proteins have an effect on HCV that is independent from miR-122.

One possibility is that DDX6 supports HCV translation. We show data indicating that DDX6 knockdown reduced HCV translation and supported the findings of Sheller et *al*. that DDX6 supports HCV translation (141). These finding contradicted those of Jangra *et al*. who did not observe an impact of DDX6 on HCV translation (259). However when we initially conducted these experiments several months earlier we found, similarly to Jangra *et al.* that DDX6 knockdown did not affect HCV translation. In both cases, our experiments were well controlled and repeated many times. Thus, we concluded that the difference we observed between current experiments and past was the state of the cells given their passage. This conclusion might also explain the contradictory results in the literature on the role of DDX6 on HCV translation.

Lastly, our data showed that DDX6 silencing had no effect on miRNA gene suppression and did not support the conclusions made by Chu *et al.* who found that DDX6 was required for miRNA mediated gene suppression (153). The sole difference between our experiments and Chu et al., was the cell types used (HeLa vs. Huh7.5), which suggest DDX6 may behave differently in different cells or there is a pathway in Huh7.5 which renderers DDX6 redundant in its ability to enhance miRNA suppression.

4.2 Several p-body proteins, including DDX6 support the HCV life cycle, but physical p-body structures do not

The identification that DDX6 promotes HCV replication and translation, but not through facilitating miR-122, suggests that p-body proteins have other roles in the HCV life cycle. As stated in the literature review and as a rationale for my original hypothesis, DDX6 is required for p-body formation; therefore it is possible that the p-body structures are required for HCV replication and translation. While our work regarding DDX6, miR-122 and HCV was being conducted, Pérez-Vilaró *et al.* demonstrated that the physical p-body structures were not required for HCV replication (257). This conclusion was made since silencing Rap55, a gene required for p-body assembly, abolished p-bodies but had no affect on HCV replication. Similarly Ariumi *et al*., and Pager *et al*. noted that over the course of HCV infection, the presence of p-bodies decreased (258) and that as p-bodies disappeared, many of the resident p-body proteins including DDX6 were recruited to lipid droplets and co-localized with core. The co-localization of DDX6 and other p-body associated proteins with the core and lipid droplets suggests that they may directly participate in many aspects of HCV life cycle such as: the formation of a replication complex, genome amplification, RNA segregation and/or virion assembly. However, a direct role for DDX6 and p-body proteins in HCV life cycle has not yet been demonstrated.

The evidence that p-bodies are not required for HCV replication suggests that DDX6 is directly involved in HCV replication or indirectly affects HCV through a mechanism distinct from p-bodies and miRNA mediated gene suppression. Jangra *el*

al. found that DDX6 interacted with HCV RNA and HCV core protein suggesting a direct role for DDX6 in the HCV life cycle. In addition, DDX6 may interact with HCV RNA through core binding since DDX6 co-immunoprecipitated with full-length HCV RNA, but did not co-immunoprecipiate in cells infected with a sub-genomic HCV replicon, which do not produce core. However, this interaction does not appear to be important for the influence of DDX6 on HCV RNA amplification since replication of HCV sub-genomic RNA is also impeded in DDX6 silenced cells. Through the use of DDX6 mutants, Jangra *et al.* also demonstrated that DDX6 helicase domain was required for HCV replication but not its interaction with core (259). The role DDX6 helicase activity plays in HCV replication is unknown, but since Jangra *et al.* reported, that DDX6 does not bind HCV without core, DDX6 helicase activity must affect HCV replication and translation indirectly. This could be by assisting in the folding or translation of a protein required to directly interact with HCV. The observation that DDX6 interacts with HCV in conjunction with core suggests that DDX6 may directly assist in HCV assembly.

4.3 Future directions for the field of HCV, p-bodies and miR-122

We originally hypothesized that DDX6 would affect miR-122 augmentation of HCV based on evidence in the literature that DDX6 was required for miRNA gene suppression activity. However, we found no relationship between miR-122, DDX6 and HCV, and found that DDX6 did not affect miRNA suppression activity in our hands. Thus, it seemed clear that the role of DDX6 is a in a different cellular function and not miRNA suppression. Since the focus of our laboratory is miRNAs and not p-bodies we focused our subsequent research into better understanding the relationship between miR-122 and HCV.

5.0 THE miR-122 BINDING REGION OF HEPATITIS C VIRUS IS SUCEPTIBLE TO SMALL INTERFERING RNA AND REPRESENTS A TARGET FOR A NOVEL ANTI-VIRAL THEREAPY

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5.1 Authors' contribution

Adam Huys performed all the experiments described and reported in the manuscript. Adam Huys wrote the manuscript, which was edited by Joyce A. Wilson.

5.2 Abstract

Hepatitis C virus (HCV) has a unique relationship and dependency on the liver specific miRNA, miR-122. Robust virus replication requires miR-122 to bind to two tandem-binding sites located within its 5' untranslated region (UTR), which is highly conserved among HCV genotypes. The activity of miR-122 on the HCV life cycle also requires incorporation of the miRNA into the host protein Ago2 and binding of this complex to the viral genome. In this study, we demonstrate that the conserved nature of the miR-122 binding region, and the accessibility of this region to Ago2/miR-122 complexes make it susceptible to siRNA targeted cleavage. Our data indicates that both sub-genomic and full-length HCV RNA replication in Huh7.5 cells is potently impeded by the presence of siRNAs that target the miR-122 binding region and we propose this as a novel HCV therapeutic strategy. Interestingly, the siRNAs designed to target the miR-122 binding region maintain their potency after several rounds of treatment, even though the presence of mutations within the 5'UTR are detectable by sequence analysis. Incorporation of point mutations within the miR-122 binding sites suggests that HCV may be able to grow in the absence of miR-122 binding but we speculate that the mutant viruses will have reduced fitness. One virus mutant, containing a point mutation in the first miR-122 binding site, was tested for growth and resistance to the miR-122 targeting siRNAs. The mutant virus replicated less efficiently than WT virus, but interestingly some of the siRNA targeting the miR-122 binding region remained equally functional at inhibiting HCV replication, even though the siRNA sequence did not match the virus genomic sequence perfectly. This suggests that the siRNAs may have a second mechanism to inhibit HCV replication besides classical siRNA target cleavage, which may impede miR-122 binding. The possibility that siRNA targeting the miR-122 binding region have dual inhibitory functions supports further study and their use as a HCV therapy.

5.3 Introduction

Hepatitis C virus (HCV) is a 9.6kb genome, positive sense RNA virus that uniquely relies on the liver specific miRNA, miR-122, for robust replication. The classical mechanism of action of miRNA conveys that miRNA bind to the 3' end of an mRNA transcript and suppresses translation. However in the context of HCV, miR-122 binds to the 5' end of the viral genome and promotes viral replication and translation (105, 110, 268). The 5' untranslated region (UTR) of HCV encodes two miR-122 binding sites that are separated by eight or nine nucleotides, and these sites are highly conserved amongst HCV genotypes (104). Mutations within the miR-122 binding sites greatly inhibit HCV replication and translation, but robust replication can be re-established with addition of synthetic miR-122 containing compensatory mutations, demonstrating miR-122 binds directly to HCV genome (104, 105).

The mechanism by which miR-122 enhances HCV replication and translation is not fully understood. The miR-122-HCV interaction requires the RNA induced silencing complex (RISC) associated protein, Argonaute 2 (Ago2) (111, 114) and its direct binding leads to the stabilization of the genome, in part by protecting HCV from host 5' exonuclease degradation by Xrn1 (107, 113). However, since knockdown of Xrn1 does not reinstate replication of HCV genomes to which miR-122 binding has been abolished, then protection from Xrn1 does not account for the observed augmentation of HCV replication attributed to miR-122 (110). Another possible way that miR-122 promotes HCV replication is by modulating the secondary structure of the HCV RNA genome. Some *in-vitro* experiments provide evidence that miR-122 binding modifies RNA structures in and near the 5'UTR (115, 118, 120). However, the role for a conformational change has not been confirmed during virus replication, and in one case (118) their role is questioned by the fact that the HCV sequences involved are absent from HCV sub-genomic replicons that still respond to miR-122. In spite of its unknown mechanism of action, miR-122 augmentation of HCV replication and the existence of conserved miR-122 binding sites are a promising target for antiviral development.

HCV inhibition by using miR-122 antagonists that bind and sequester miR-122 effectively inhibit HCV in cell culture, in Chimpanzees, and have provided encouraging results in human clinical trials (103, 243). In HCV infected humans, miR-122 antagonist treatment decreased HCV viral loads at all doses, and at the highest dose used, reduced HCV titers to undetectable levels. HCV titres rebounded following treatment cessation and longer treatment durations are currently being tested. The only side effect of the treatment was an anticipated decrease in serum cholesterol levels (103). Due to a possible role for miR-122 as a tumour suppressor, longer trials must be done with caution, and long-term treatment with miR-122 antagonists is not advisable. Importantly, miR-122 antagonist treatment did not select for HCV genomes having point mutations to the miR-122 binding sites thus, it appears to not select for viruses that have escaped reliance on miR-122. Therefore, miR-122 antagonism may be a treatment with a high barrier to the development of resistance.

Given the dependence of HCV on miR-122, the early reported success of miR-122 antagonist as a treatment, the knowledge that Ago2 loaded with miR-122 can access the 5'UTR, and the conserved nature of the miR-122 binding region, we speculated that the miR-122 binding region within the 5'UTR of HCV constitutes a potential target for siRNA directed cleavage. This speculation is further supported by the pharmacokentic study by Laxton *et al.* suggesting that HCV can be inhibited by using antisense oligonucleotides targeting the 5' terminus of the HCV genome, including the miR-122 binding region (269). Previous attempts to use siRNA directed cleavage at conserved regions of the HCV genome displayed a high level of success (244-246), but over the course of several treatments their potency began to decrease. siRNA targeted cleavage of target RNAs requires perfect sequence match between the siRNA and the target. The decrease in effectiveness of HCV inhibiting siRNA was attributed to the evolution of escape mutations having nucleotide mutations within the siRNA target sequences that conferred resistance to inhibition (247, 248). This illustrates the importance of targeting conserved and functionally important sequences to limit the possibility of evolution of resistance

In this study, we provide evidence that siRNAs designed to target the miR-122 binding region can inhibit HCV replication in cell culture. We hypothesized that it would be difficult for HCV to escape miR-122 binding-site targeting siRNA due to the conserved nature and essential function of this region and as expected the siRNAs continued to be effective after several rounds of treatment. However, sequence analysis did detect escape mutants but we show that a mutant virus, containing a point mutation in first miR-122 binding site does not abolish inhibitory activity of some of the siRNAs.

5.4 Material and methods

5.4.1 Plasmids

The JFH-1 FLuc sub-genomic replicon plasmid (pSGR JFH-1 Fluc) was kindly provided by Dr. T. Wakita (262). The plasmid pJ6/JFH-1 RLuc (p7-RLuc2A) encoding a full-length HCV genome expressing a Renilla luciferase (Rluc) gene directly downstream of the p7 gene, (263), and pFLneo-J6/JFH-1(p7-Rluc2a) encoding a full-length bicistronic HCV replicon RNA expressing neomycin from the HCV IRES and Rluc within the full-length HCV polyprotein, directly downstream of the p7 gene, were provided by Dr. C. M. Rice (herein called pJ6/JFH-1 RLuc and pJ6/JFH-1 Neo Rluc respectively). The miR-122 site 1 position 3 mutant sub-genomic replicon (pSGR JFH-1 Fluc S1:P3) was generated as described previously (116), and pJ6/JFH-1 site 1 position 3 plasmid (J6/JFH-1 RLuc S1:P3) was generated by digesting SGR S1:P3 with *Age*I and *Eco*RI to isolate the 5'UTR and then inserting into a partially digested J6/JFH-1 Rluc plasmid. The sub-genomic replicon plasmid having both neo and luciferase genes, pFK I389 lucubineo NS 3-3' JFH-1 (herein called SGR lucubineo JFH-1 Fluc) was kindly provided by Dr. V. Lohmann. pLuc JFH-1 5'UTR x 2, a miR-122 suppression activity reporter plasmid having two HCV 5' UTR sequences inserted downstream of a luciferase gene, was created by making two point mutations in pLuc-122x2, a gift from Dr, P. Sarnow (104). Briefly, pLuc-122x2 was designed by cloning two 5'UTR of an HCV H77 construct behind a firefly

luciferase. The sequence of the H77 genotype inserts were modified to that of the JFH-1 genotype by using quick change mutagenesis and the primers, 5' GCGACACTCCGCCATGAATCA and 5' TGATTCATGGCGGAGTGTGTCGC to generate the plasmid pLuc JFH-1 5'UTR 1st. The A to G swap required for the second UTR was achieved by digesting pLuc122 x 2 with *Eco*R1 and *Sal*I to isolate the second UTR. Following ligation into BluescriptKS(+) and mutagenesis using the methods described above, the mutated second UTR, now having the JFH-1 sequence, was cloned into plasmid pJFH-1 5'UTR 1st, generating plasmid pLuc JFH-1 5'UTR x 2. Plasmid pT7 and pRL-TK plasmids were obtained from Promega Co. (Madison, WI, USA)

5.4.2 Cell culture

The human hepatoma cell lines Huh7.5, and Hep3B were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 0.1nM non-essential amino acids (Wisent, Montreal, Canada) and 100µg/ml Pen/Strep (Invitrogen, Burlingtion ON, Canada). Lunet-Lucubineo JFH-1 cells (270), provided by Dr. V. Lohmann, were grown as described above in the presence of 800µg/ml G418 Sulfate (Wisent, Montreal, Canada) to maintain the HCV replicon. Huh7.5 cells harbouring J6/JFH-1 Neo Rluc were provided by Dr. Liu Qiang (VIDO-InterVac, Saskatoon, Saskatchewan, Canada) and were cultured identically to Lunet-Lucubineo JFH-1 cells. For generating, maintaining and selecting cells containing HCV replicons, 800µg/ml G418 Sulfate was added to supplemented DMEM.

5.4.3 Small interfering RNAs (siRNA) design and sequence

The siRNAs designed which target the miR-122 binding regions of HCV were constructed using various software available on-line and the target sequence were as follows: siRNA18-36 GCGACACUCCGCCAUGAAU, siRNA19-37 CGACACUCCGCCAUGAAUC and siRNA21-43 ACACUCCGCCAUGAAUCACUCCC. The sequence of siRNA JFH-1 6367 was adapted from the highly effective siRNA

described previously to inhibit HCV con1 genotype, by modifying the sequence to match the same region in JFH-1 GACCCACAAACACCAAUUCCC (246). The control siRNA (siControl) target sequence was GAGAGUCAGUCAGCUAAUCA as was used in a previous study (111). All the siRNAs were synthesized by Thermo-scientific (Lafayette, CO).

5.4.4 *In-vitro* RNA transcription

HCV RNA was synthesized using the MEGAScript T7 High Yield *in-vitro* Transcription Kit (Life Technologies, Burlington, ON, Canada). Firefly (Fluc) and Renilla (RLuc) messenger RNA (mRNA) where transcribed using a mMessage mMachine T7 Transcription Kit (Life Technologies, Burlington, ON, Canada). The transcription process was conducted using the suggested manufacture's protocol. In a process described previously (116), *Xba*I linearized plasmid was used as the transcription template for HCV constructs and *Xmn*I linearized pT7 luciferase and *Bgl*II linearized pRL-TK were used as templates for Fluc and RLuc mRNA transcription, respectively.

5.4.5 Non-HCV siRNA knockdown assay

On Day 0, 6.5 x 10^4 Hep3B cells/well were plated in a 24 well dish and incubated overnight. The following day Hep3B cells were transfected with 100ng of pRL-TK and pLuc JFH-1 5'UTR x 2, along with 0.1pmol of an siRNA and 1 µl lipofectamine 2000 (Life Technologies, Burlington, ON, Canada). The transfection mixture was prepared according to the suggested manufacture's protocol. The cells were slowly rocked for 20 minutes at room temperature before being incubated at 37°C, 5% CO_2. On day 2, the cells were lysed using passive luciferase lysis buffer (Promega).

5.4.6 Electroporation of Huh7.5 cells

Huh7.5 cell were electroporated as previously described (116).

5.4.7 Transient HCV replication assay

Briefly, Huh7.5 cells were co-electroporated with varying amounts of HCV RNA, 60 pmol of siRNA and 1µg of control mRNA. Samples were plated and harvested for luciferase expression at 2 hour, and days 1-3. 1µg of HCV was used in assays conducted with J6/JFH-1 RLuc (p7-RLuc2A) and 5µg of RNA was used in all the other replication assays.

5.4.8 Escape mutant selection assay

Huh7.5 cells harbouring J6/JFH-1 Neo Rluc and Lunet-Lucubineo JFH-1 cells were electroporated with 60pmol of a specific siRNA. After electroporation, a small portion of the cells was plated for harvest and luciferase assay, and the remaining cells were cultured in the presence of G418. After two weeks, ¼ of the cells were harvested in Trizol, ¼ were cryofrozen, and ½ were electroporated with 60pmol of the same siRNA used in the previous electroporation and the process was repeated. The cells continued in this cycle until they had been electroporated with the same siRNA seven times. For each siRNA, three independent selections were done unless otherwise noted.

5.4.9 Luciferase assay

Cells were washed two times in Dulbecco's PBS then lysed with 100µl of passive luciferase lysis buffer (Promega). Luciferase levels within the lysate were measured by using Firefly, Renilla, or Dual luciferase kits (Promega) and light emission was measured by using a Glomax 20/20 Luminometer (Promega). The luciferase assays were performed as suggested by the manufacturer's protocols.

5.4.10 RNA purification

Cells were harvested into 1ml of Trizol and total cellular RNA was isolated using the suggested manufacturer's protocol.

5.4.11 Sequencing of the miR-122 binding region of HCV 5'UTR

Purified RNA was converted to cDNA using iScript select cDNA synthesis kit (Bio-Rad Inc., Missassauga, On, Canada) and the manufacturer's recommended protocol with the addition of a specific J6/JFH-1 Neo Rluc reverse transcription primer 5'TGTTGTGCCCAGTCATAGCCC. The cDNA was then amplified using Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA) and the suggested manufacturer's PCR protocol. The primers used for the PCR reactions were: forward primer 5'GAATTCTAATACGACTCACTATAGACCTGCCCCTA ATAGG and reverse primer 5'GAACCTGCGTGCTGCAATCCATC. The forward primer was designed to anneal directly to the 5' terminus of the HCV genomic RNA and contains additional sequence of an *Eco*R1 site and a T7 promoter. The PCR product was gel purified using Qiaquick Gel Extraction Kit (Toronto, ON, Canada). The purified PCR product was ligated into pCR™-Blunt II-TOPO® vector using Zero Blunt TOPO PCR Cloning Kit (Invitrogen) according to the manufacturer's recommended protocol, then electroporated into electro-competent TOP-10 cells and incubated overnight 37°C on LB + 50μg/ml Kanamycin plates. The next day, individual colonies were picked and sent for Templiphi amplification and Sanger sequencing (GE Healthcare, National Research Council of Canada's Plant Biotechnology institute sequencing core, Saskatoon SK). Sequences were derived from 3 independent resistance selections for si18-36 treated cells, 2 independent experiments from si19-37 treated cells and 1 experiment from both siControl and siJFH-1 6367 treated cells.

5.4.12 Analysis of sequencing data

All sequences where analyzed using Clone Manager software.

5.4.13 Statistical analyses

Data are presented as the average of at least three independent experiments unless otherwise indicated. Data analysis was carried out with Prism 6 software. P values, unless otherwise indicated were calculated by using a Student *t*-test, *P<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.5 Results

5.5.1 siRNA designed to specifically target the miR-122 binding region within the 5'UTR of HCV are capable of functioning as siRNA

With the knowledge that miR-122 accesses the HCV 5' UTR in association with Ago2 to promote HCV replication we speculated that siRNA/Ago2 complexes targeting the miR-122 binding site region will also have access to this region and cleave the HCV genomic RNA. In addition, this region is predicted to have limited secondary structure, another feature that promotes access of siRNA/Ago2 complexes (115). Using standard prediction algorithms, we designed 3 siRNAs, si18-36, si19-37 and si21-43 to target the miR-122 sites within the HCV 5' UTR (Fig. 5.1A).

To test the siRNAs ability to form an RNA induced silencing complex (RISC) and knockdown mRNA, we tested their ability to knockdown a luciferase mRNA having the HCV miR-122 binding region within its 3' UTR. The siRNAs were co-transfected into Hep3B cells with a firefly reporter plasmid containing two binding sites specific for the miR-122 binding region siRNAs downstream of a firefly luciferase gene, and a Renilla expressing transfection control plasmid (Fig. 5.1B: left panel). We used Hep3B cells because they do not express detectible miR-122 to

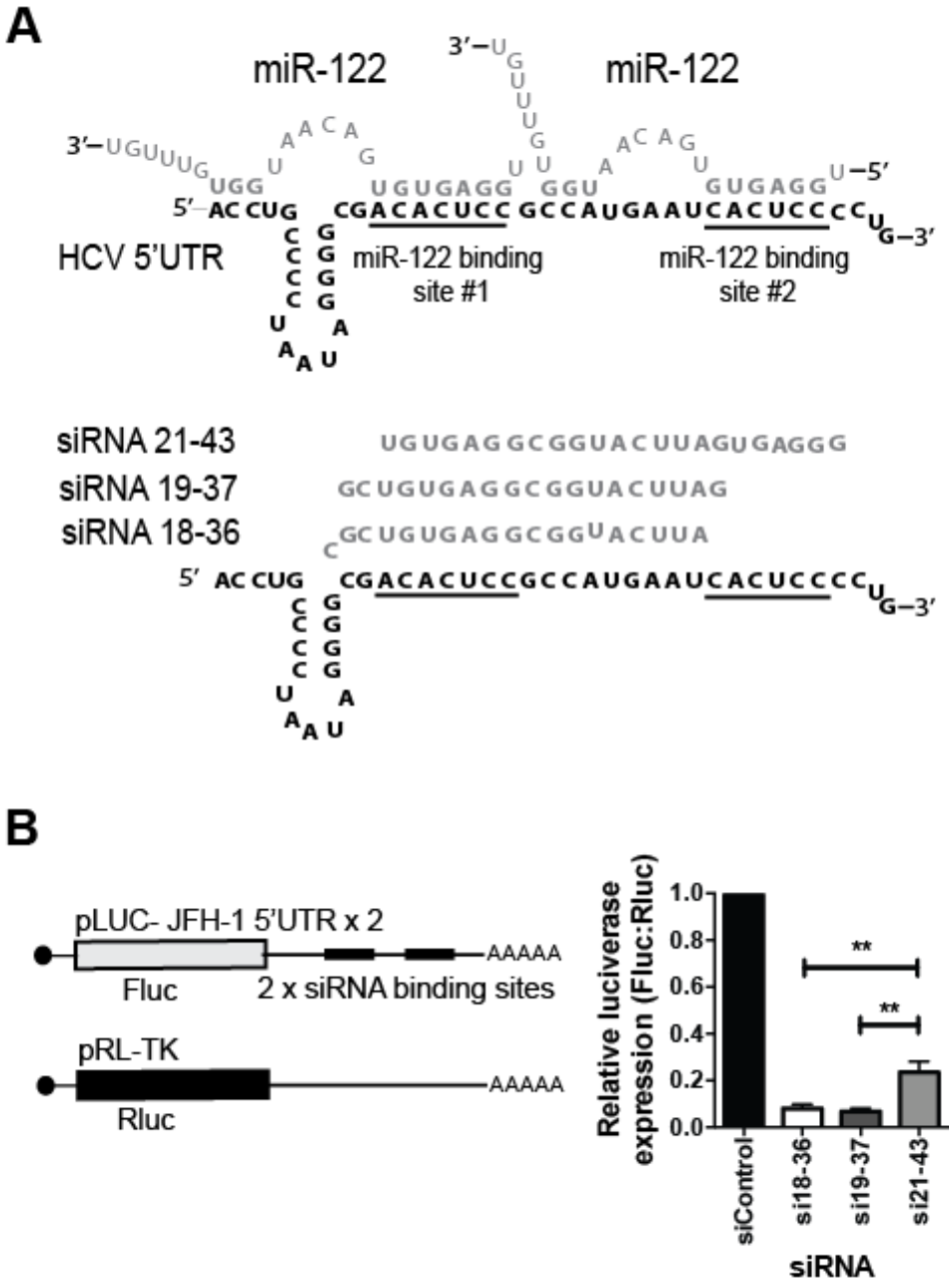


Figure 5.1 siRNA target sites in the miR-122 binding region and ability of the siRNAs to function as a conventional siRNA. (A) A schematic diagram of how miR-122 bind the 5'UTR of HCV JFH-1 and the locations of 3 siRNA (si18-36, si19-37, and si21-43) that target the miR-122 binding region of the HCV JFH-1 5' UTR. (B) The effectiveness of the individual siRNA to decrease luciferase expression by binding to two seed sequences located downstream of a firefly luciferase gene. Data in B represents the average of four independent experiments, and significance was determined by performing a one-way ANOVA with Tukey's multiple comparisons test.

remove any confounding effects of exogenous miR-122 binding to the target sites. The siRNAs si18-36, si19-37 and si21-43 decreased firefly luciferase expression by 92%, 93% and 76% respectively compared to the cells transfected with control siRNA (siControl) after normalization to the Renilla transfection control plasmid (Fig. 5.1B: right panel). These results suggest that the designed siRNA, si18-36, si19-37 and si21-43 have the potential to form a RISC and can bind to and knockdown mRNAs containing the HCV 5' UTR and miR-122 binding sequences, and that si21-43 is less effective than the others.

5.5.2 Sub-genomic and full-length HCV replication is decreased in Huh7.5 cells after treatment with si18-36, si19-37, and si21-43

To assess the ability of the siRNAs designed to target the HCV 5'UTR to block HCV replication, Huh7.5 cells where co-electroporated with J6/JFH-1 Rluc (Fig. 5.2A), or SGR JFH-1 FLuc (Fig. 5.3A) and one of the siRNAs: si18-36, 19-37, si21-43 or a negative control siRNA (siControl). A positive control siRNA, JFH-1 6367 similar to the one shown previously to efficiently knockdown HCV by targeting the NS5B region of HCV was also used (246). The amount of luciferase generated from the HCV construct was used as a reporter to reflect HCV replication. All three siRNAs decreased HCV replication in cells that received full-length J6/JFH-1 Rluc RNA. Cells co-transfected with J6/JFH-1 Rluc and siRNAs had reduced luciferase production compared to the cells treated with siControl (Fig. 5.2B). si18-36 inhibited luciferase expression on days 1, 2 and 3 by 93%, 92% and 80% respectively. si19-37 reduced HCV replication by 74%, 66%, 50% respectively. si21-43 treatment led to a decrease in luciferase expression by 58%, 60%, and 44% respectively. siJFH-1 6357 led to a decrease in HCV of over 97% at all three days (Fig. 5.2C). These observations were similar to those collected when using the sub-genomic HCV RNA construct (Fig. 5.3B). si18-36 decreased HCV replication by 91%, 84%, and 64% on days 1, 2, and 3 respectively. si19-37 treatment resulted in a decrease in luciferase expression by 80%, 68% and 44% on days 1, 2, and 3 respectively. si21-43 inhibited HCV replication by 57%, 52%, and 35% on days 1, 2 and 3 respectively. siJFH-1 6367, as

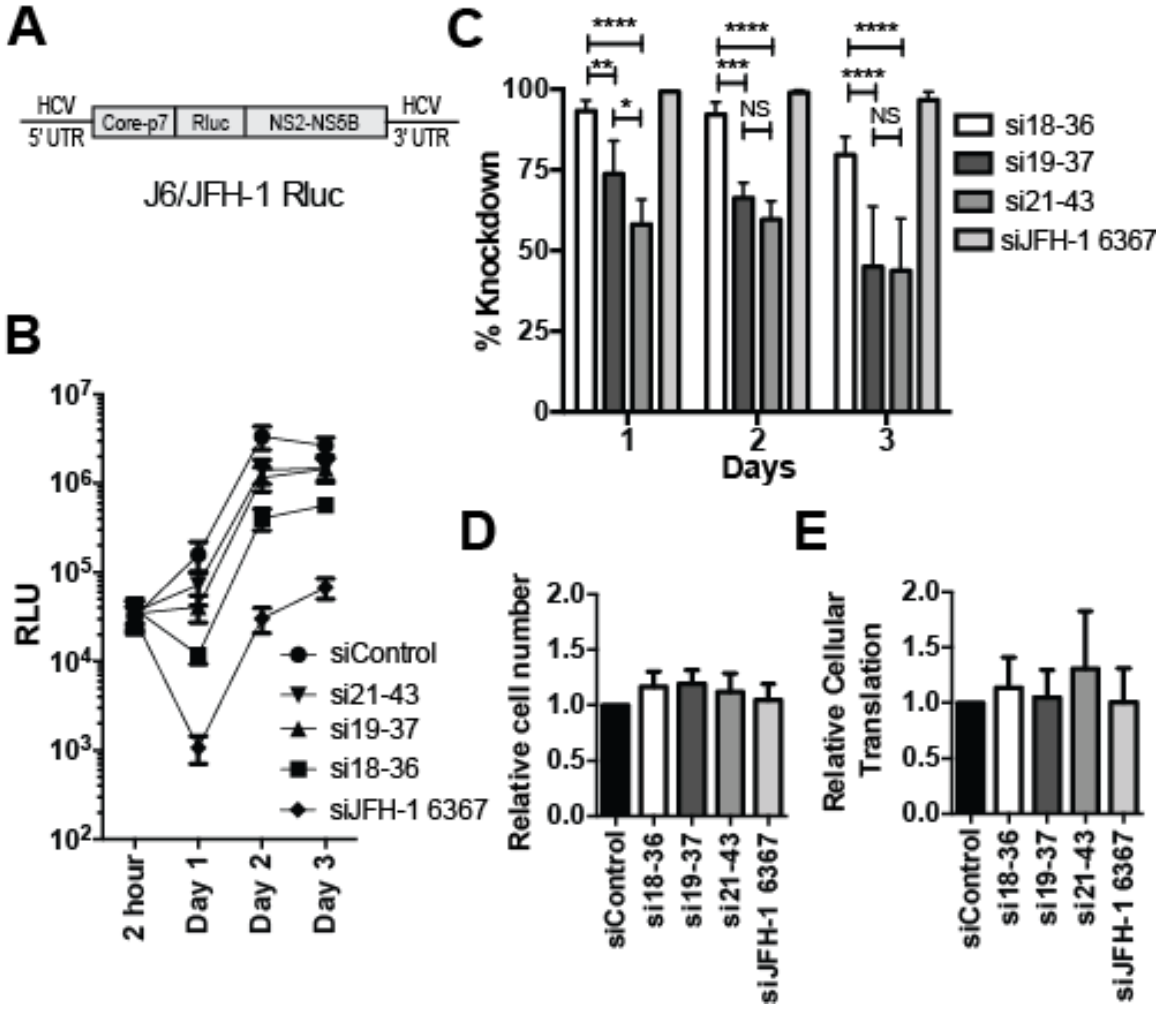


Figure 5.2 siRNA knockdown of full-length HCV J6/JFH-1 RLuc RNA. (A) A schematic diagram illustrating full-length J6/JFH-1 RLuc RNA (B) Renilla luciferase expression by the HCV RNAs at the indicated time points in Huh7.5 cells co-electroporated with J6/JFH-1 RLuc and either a control siRNA (siControl), an siRNA designed to target the miR-122 binding region (si18-36, si19-37 or si21-43) or a siRNA specific for the NS5B coding region (JFH-1 6367). (C) The percent reduction in Renilla luciferase expression induced by the siRNAs relative to the control siRNA (siControl). (D) The relative cell numbers (E) and levels of firefly luciferase expressed from a transfection control mRNA in cells that were treated with various siRNA. All data represents the average of five independent experiments and the error bars in B represent standard error of the mean, while the others represent standard deviation. Significance was determined by performing a two-way ANOVA with Tukey's multiple comparisons test.

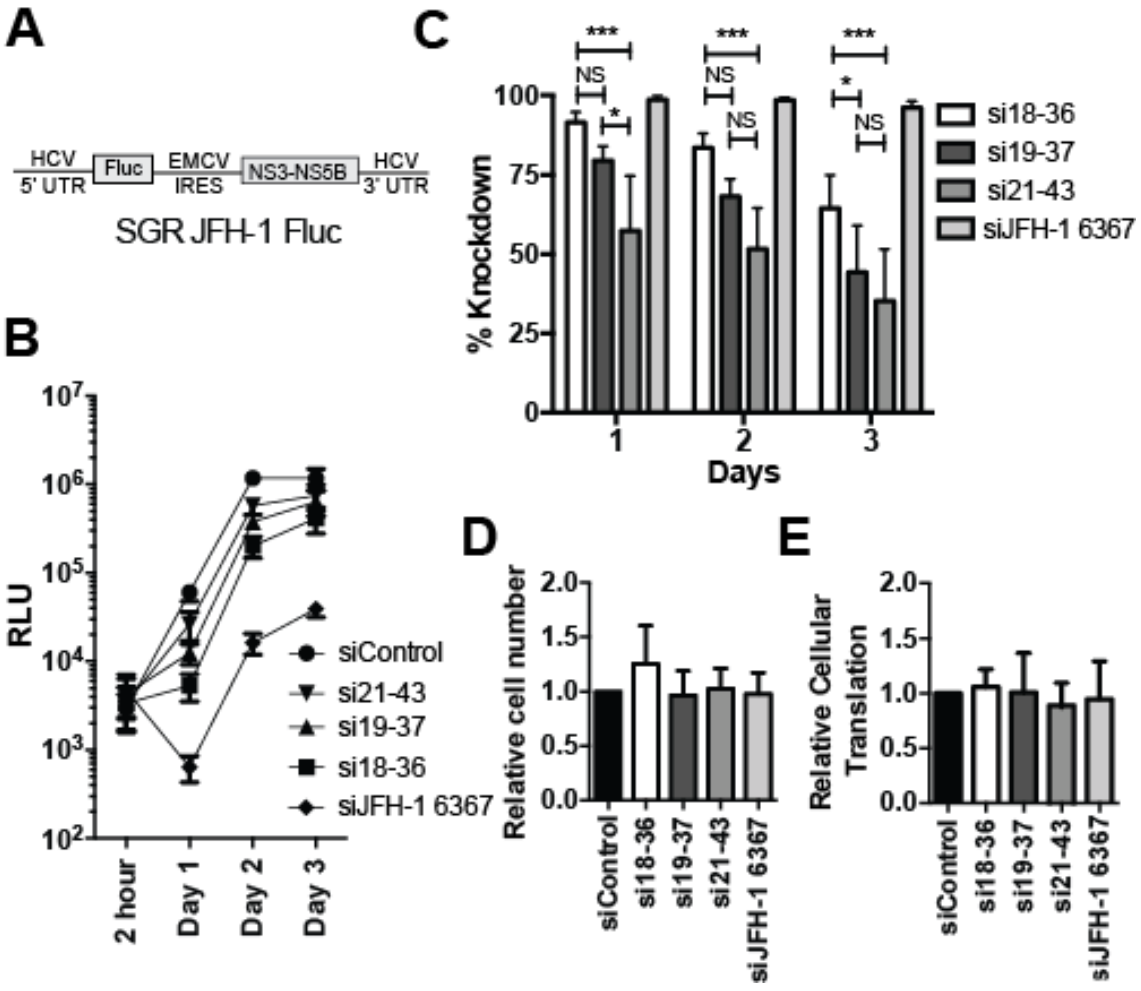


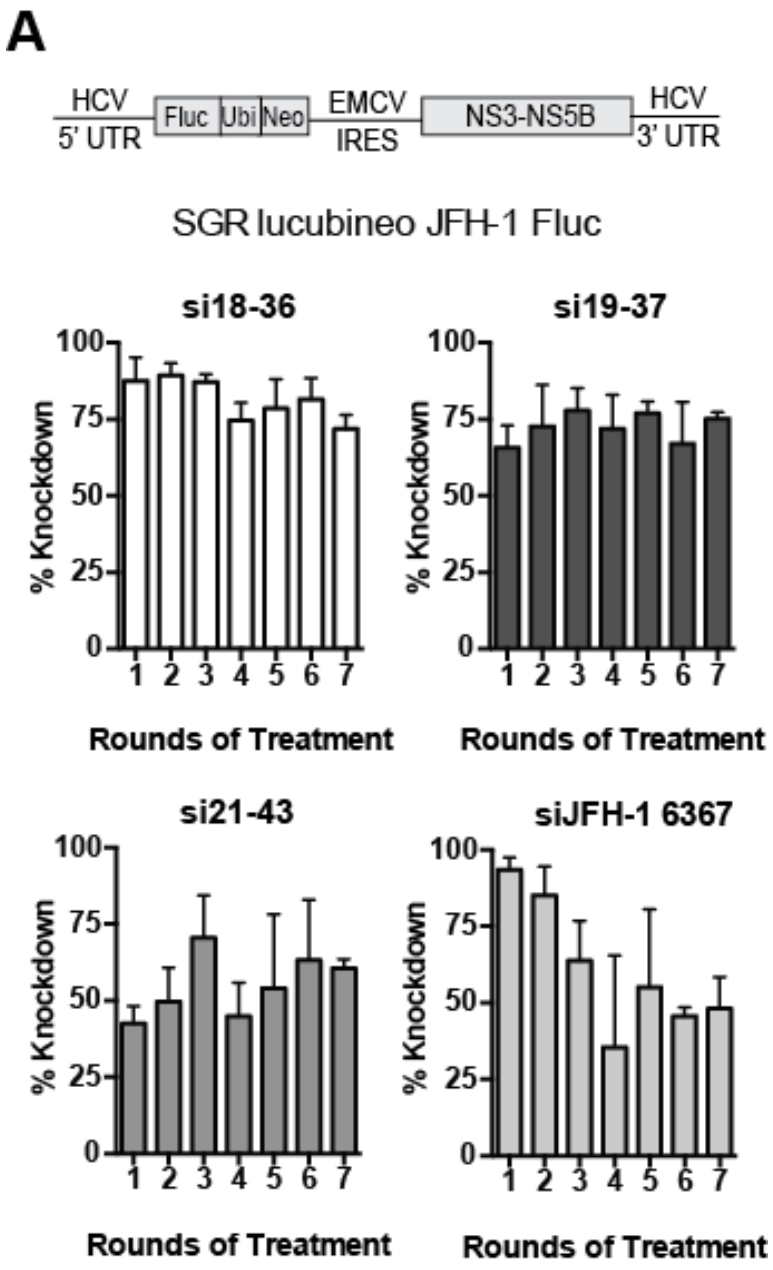
Figure 5.3 siRNA knockdown of HCV sub-genomic RNA. (A) A schematic diagram illustrating the SGR JFH-1 FLuc replion RNA. (B) Firefly luciferase expression at the indicated time point by Huh7.5 cells co-electroporated with SGR JFH-1 Fluc and either the siRNA si18-36, si19-37, si21-43, JFH-1 6367 or a control siRNA (siControl). (C) The relative percent reduction in firefly luciferase expression induced by siRNA compared to cells treated with siControl. (D) The relative cell numbers (E) and levels of Renilla luciferase expressed from a transfection control mRNA in cells that were treated with various siRNA. All data represents the average of four independent experiments and the error bars in B are represented as standard error of the mean, while the others are represent by standard deviation. Significance was determined by performing a two-way ANOVA with Tukey's multiple comparisons test.

expected, decreased HCV replication by over 96% during the three-day time course (Fig. 5.3C). The treatment of Huh7.5 cells with the siRNAs did not affect cell viability or normal cellular translation measured from a co-electroporated control luciferase mRNA (Fig. 5.2D and E, Fig. 5.3D and E). The ability of si18-36, si19-37 and si21-43 to decrease luciferase expression from a full-length construct and SGR indicate that the siRNAs designed to target the 5'UTR of HCV inhibit HCV replication.

5.5.3 Cell lines stably harbouring HCV constructs remain susceptible to siRNAs targeting the 5'UTR after numerous rounds of treatments

Due to the error prone nature of HCV RNA dependent RNA polymerase, HCV rapidly develops resistance to antiviral treatments. One such example of this is HCV's ability to escape inhibition by siRNA siJFH-1 6367 after several rounds of treatment (247). In an effort to determine whether HCV can escape inhibition by siRNA targeting it's 5'UTR, we used a positive selection method to identify escape mutants. For this selection, cells harbouring HCV constructs carrying both luciferase and neomycin resistance genes, SGR lucubineo JFH-1 Fluc (Fig. 5.4A: upper panel) and FL J6/JFH-1 Neo Rluc (Fig. 5.4B: upper panel) were treated with the siRNA to knockdown the HCV RNA, and then neomycin selection was used to select for only cells in which viral RNA remained. The siRNA knockdown efficacy was assessed after each treatment by measuring luciferase gene expression compared to the cells treated with siControl and the knockdown/selection method was repeated 7 times to allow selection of resistant mutants. The knockdown efficiency at each round of treatment was compared with the original treatment effectiveness to assess evolution of treatment resistant HCV. With each round of treatment, the effectiveness of the positive control, siJFH-1 6367, on SGR lucubineo JFH-1 Fluc began to wane, generally trending downwards after the second round of treatment, suggesting the generation of resistance to siJFH-1 6367 (Fig. 5.4A, lower right panel). However, si18-36, si19-37 and si21-43 treatments did not demonstrate a decline in effectiveness against SGR lucubineo JFH-1 (Fig. 5.4A: middle and lower left panels). Similarly, the treatment of JFH-1 Neo Rluc with siJFH-1 6367 resulted

Figure 5.4


A
HCV
5' UTR
Fluc
Ubi
Neo
EMCV
IRES
NS3-NS5B
HCV
3' UTR
SGR lucubineo JFH-1 Fluc
si18-36
si19-37
si21-43
siJFH-1 6367
% Knockdown
100
75
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1 2 3 4 5 6 7
Rounds of Treatment

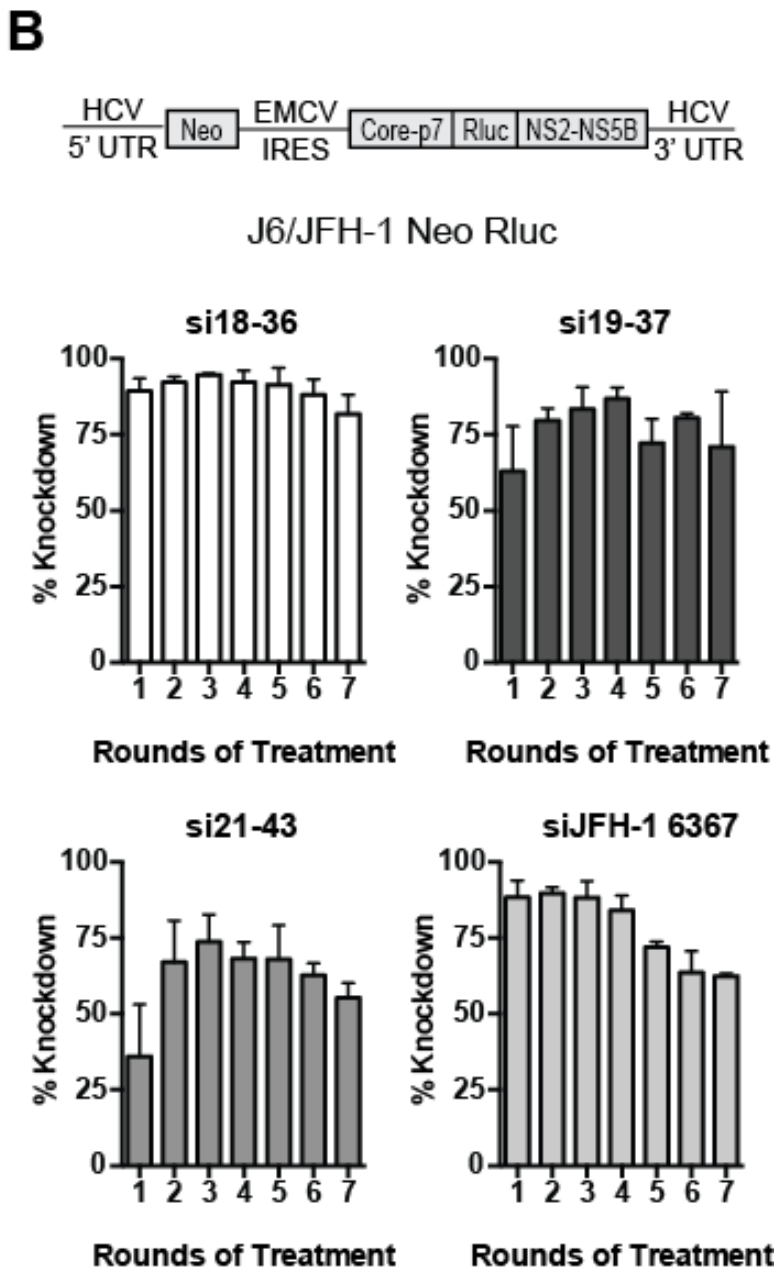
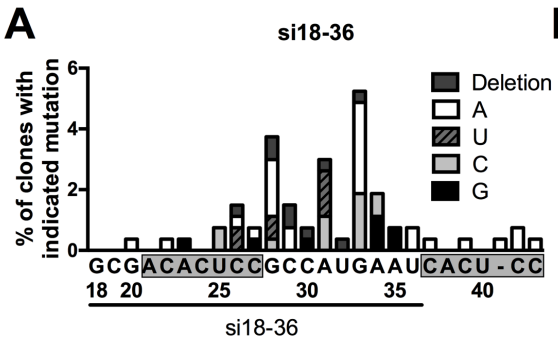


Figure 5.4 Luciferase expression levels during selection of siRNA resistant escape mutants. A schematic illustration of the SGR lucubineo JFH-1 FLuc replicon RNA (A, upper panel), and J6/JFH-1 Neo Rluc (B, upper panel). The bar graphs represent the % reduction in luciferase expression from SGR lucubineo JFH-1 (A) or J6/JFH-1 Neo Rluc (B) expressing cells following multiple rounds of treatment with the indicated siRNA compared to the same cells electroporated with a control siRNA. In all cases the data are presented as averages from 3 independent replicates of resistance selection for each siRNA, except for panel B; treatment rounds 6 and 7 of si21-43 and rounds 5 and 7 of JFH-1 6367 which represent the average of two independent experiments.

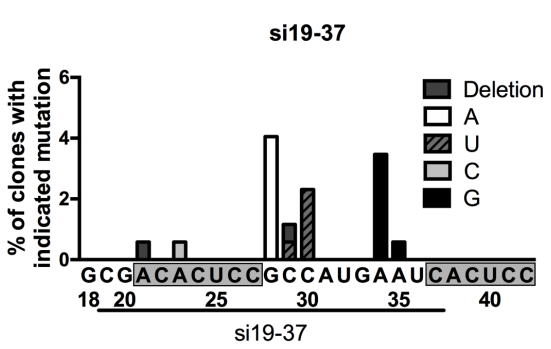
in a general decrease in effectiveness of the siRNA after several rounds of treatment (Fig. 5.4B, lower right panel) but the siRNAs targeting the 5'UTR, si18-36, si19-37 and si21-43, did not decrease in effectiveness after several rounds of treatment, nor did they demonstrate a trend that indicated the presence of resistance (Fig. 5.4B middle and lower left panels). These observations suggested that HCV does not appear to generate resistance to siRNAs targeting the miR-122 binding region.

5.5.4 The 5'UTR of constructs maintained in stable cells that have been treated numerous times with the same siRNA contain mutants within the miR-122 binding region

In spite of the fact that HCV did not appear to become resistant to siRNAs that target the 5' UTR, sequence analysis of the HCV 5'UTR from the cells that had been treated numerous times with either si18-36 or si19-37 revealed point mutations within the siRNA target sequence. In si18-36 treated cells, of the 267 UTRs sequenced, 67 displayed mutations in the siRNA target sequence, and included mutations within the first miR-122 binding site and the region between the two miR-122 binding sites (Fig. 5.5A: graph). Unexpectedly, treatment with si18-36 also produced six mutations within the second miR-122 binding site in sequences that were not directly targeted by the siRNA. Also unexpectedly, one viral clone harboured three point mutations within the second miR-122 binding site and suggests that a significant change in the miR-122 binding site can be tolerated. However, fitness analysis of the array of mutants identified remains to be done to confirm their phenotype and dependence on miR-122. (Fig. 5.5A: table). In si19-37 treated cells, 173 UTRs were sequenced and 23 contained mutations within the miR-122 binding region (Fig. 5.5B: graph). si19-37 treated cells generated fewer mutations per clone (22/173=13%) than observed in si18-36 treated cells (65/267=24%). Though the majority of the mutations identified in si19-37 treated cells were in the spacing region between the two miR-122 binding sites, this siRNA did not yield any mutations in the second miR-122 site. The most abundant mutations found in si19-37 treated cells were at position 28 and 34. These positions



Multiple mutations			
Position	Frequency	% Frequency	Mutation
22 & 28	1	0.37%	C to A & G to A
23 & 25	1	0.37%	A to G & U to C
28 & 29	1	0.37%	Deltion & C to A
30 & 36	1	0.37%	C to G & U to A
31 & 32	1	0.37%	Both deletions
31 & 35	1	0.37%	A to U & A to G
36 & 37	1	0.37%	U to A & C to A
39, 41 & 42	1	0.37%	C to A, C to A & C to A
40i & 41	1	0.37%	Insertion of A & C to A



Multiple mutations			
Position	Frequency	% Frequency	Mutation
21 & 23	1	0.58%	Deletion & A to C
28 & 34	1	0.58%	G to A & A to G
30 & 34	4	2.31%	C to U & A to G

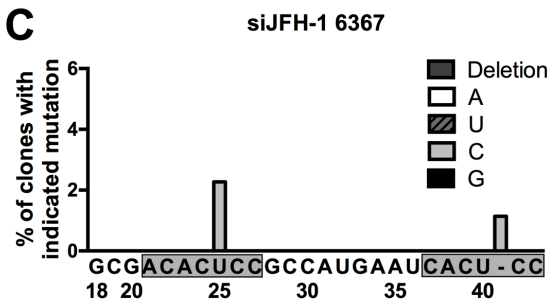


Figure 5.5 Mutants within the miR-122 binding region of the HCV 5'UTR sequence yielded after seven rounds of treated with si18-36, si19-37, or siJFH-1 6367 and selection with G418. 5'UTR sequences from HCV RNA extracted from cells harbouring HCV RNA after seven rounds of resistance mutation selection with (A), si18-26 (B), si19-37 (C) or siJFH-1 6367. The grey highlighted nucleotides represent the miR-122 binding sites, and the underlined nucleotide indicated where the siRNA binds.

are the least conserved between HCV genotypes and the mutation of G to A at position 28 is found in the genotype 1a H77 sequence, and the mutation at position 34 A to G is found in genotypes 1b and 3a (117). We also observed that many of the UTRs isolated from cells treated with si19-37 contained paired mutations within the miR-122 binding region (Fig. 5.5B: table). In positive control siJFH-1 6367 treated cells we also isolated 3 RNAs out of 88 UTRs sequenced (3%) having mutations within the miR-122 binding region, which suggests a potential growth enhancing phenotype for these mutation. However, the conversion of a U to C in siJFH-1 6367 treated cells at position 25 was only observed a few times in si18-36 and never in si19-37 treated cells. We did not detect any mutations in the miR-122 binding sites of 80 5'UTR sequenced from HCV constructs maintained in cells which had never been treated with siRNA (data not shown).

5.5.5 HCV that contains a point mutation in the siRNA targeted binding site is more susceptible to si19-37 and si21-43, while becoming more resistant to si18-36

To begin to analyze the influence of the miR-122 point mutations on HCV RNA replication fitness and susceptibility to the antiviral siRNAs, we analyzed a full-length virus bearing a point mutation at position 26. This mutation has also been termed S1:p3 in ours and other's work and in this context is called J6/JFH-1 Rluc S1:p3 (106). The point mutation is present in the 1st miR-122 binding site of HCV 5'UTR and is the result of changing a cytosine to a guanine at position 26 (Fig. 5.6A). This mutation abolishes the sequence identified between the viral RNA and all three siRNAs, si18-36, si19-37 and si21-43 (Fig. 5.1A). J6/JFH-1 Rluc S1:p3 RNA does not replicate as efficiently as WT virus (Fig. 5.2B vs. Fig. 5.6B: siControl and 1µg vs. 5µg of HCV RNA), but its replication can be reinstated by using a miR-122 mutant that reinstates binding (unpublished data) confirming that loss of miR-122 binding has an effect on virus fitness. Using this system to test the efficacy of our siRNAs, we found that, in spite of the sequence mismatch between this HCV genomic sequence and our antiviral siRNAs, they all retained antiviral activity. Surprisingly, some even

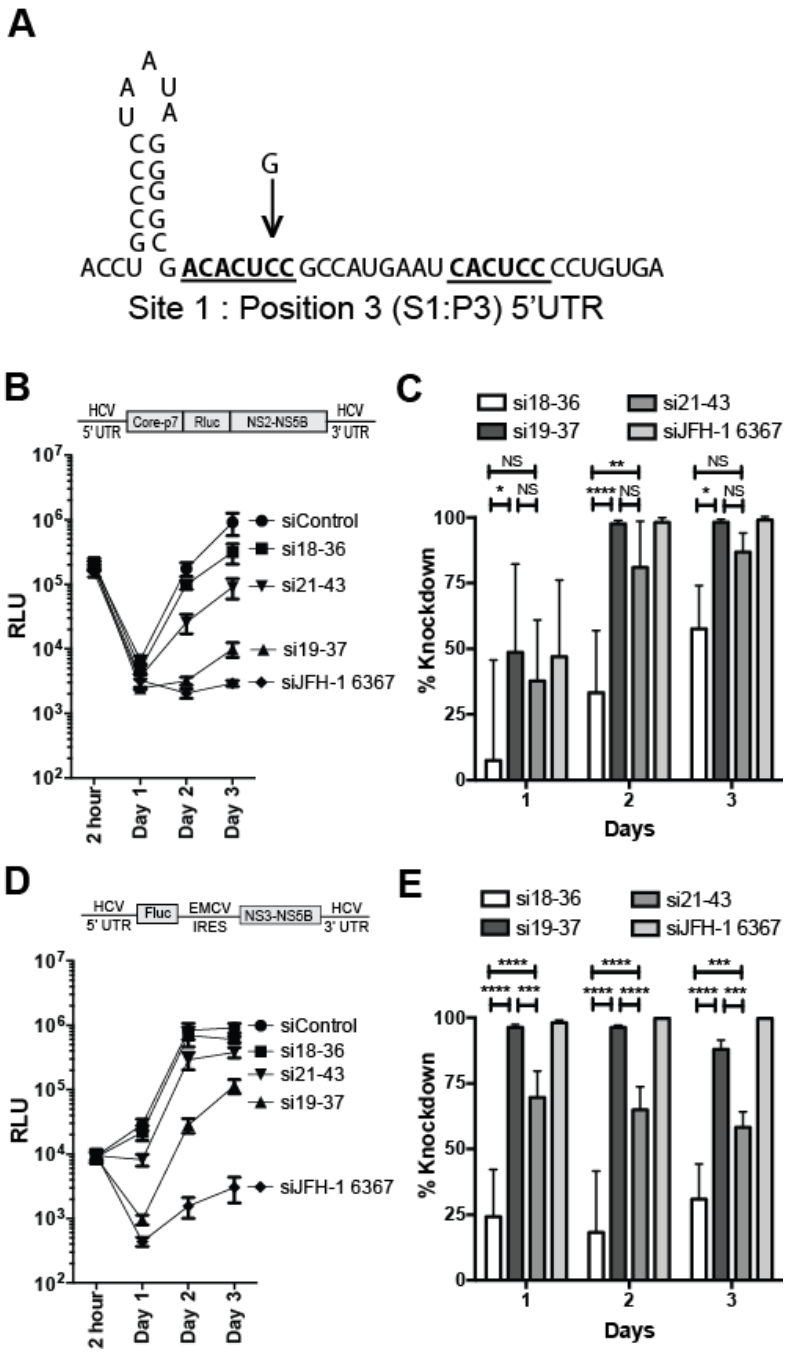


Figure 5.6 siRNA knockdown of full-length and SGR HCV RNA having miR-122 S1:p3 mutation. (A) A schematic diagram of the 5'UTR of HCV and the S1:p3 mutation. (B) The amount of luciferase expressed in Huh7.5 cells at the indicated time points co-electroporation of J6/JFH-1 Rluc S1:p3, or (D) SGR JFH-1 FLuc S1:p3 with the indicated siRNA. The relative percent decrease of luciferase expression of (C) J6/JFH-1 Rluc S1:p3, or (E) SGR JFH-1 Fluc S1:p3 relative to siControl siRNA. Significance was determined by performing a two-way ANOVA with Tukey's multiple comparisons test.

had enhanced antiviral activity (Fig. 5.6B and C). When J6/JFH-1 Rluc S1:P3 was treated with the siRNA targeting the miR-122 binding sites; si18-36 reduced luciferase expression by 7%, 33%, and 58% on days 1, 2 and 3. This siRNA was less efficient than it had been on WT HCV RNA as would be expected for an siRNA that does not match its target sequence perfectly (Fig. 5.7A: top graph). si19-37 decreased luciferase production by 40%, 95%, and 94% on days 1, 2 and 3 while si21-43 inhibited luciferase expression by 38%, 81% and 87% on days 1, 2, and 3b (Fig. 5.6B and C). Interestingly both si19-37 and si21-43 displayed greater potency towards the S1:P3 mutant virus than the WT virus. si19-37 and si21-43 inhibited S1:P3 1.4 fold and 2 fold greater then WT virus on days 2 and 3 respectively (Figure 5.7A: middle graphs). This data suggests that siRNAs 19-37 and 21-44 may use an alternative method of inhibition beyond that of siRNA directed cleavage. Similar results were seen with HCV SGR RNAs (Fig 5.6D, E and Fig. 5.7B).

5.6 Discussion

We have demonstrated that targeting the miR-122 binding region of HCV with the siRNAs si18-36, si19-37 or si21-43 inhibit HCV replication in the context of both full-length HCV RNA and a sub-genomic replicon RNA. si18-36 displayed the greatest effectiveness on WT HCV RNAs, followed by si19-37 and si21-43. Using these siRNAs we achieved knockdown efficiency of up to 93%, similar to that of the very effective siJFH-1 6367.

Due to the error prone characteristic of HCV RNA dependent RNA polymerase, HCV can readily develop resistance to antiviral agents through the selection of genomes carrying randomly generated mutations that confer drug resistance. Because miR-122 binding has such a potent role in promoting the HCV life cycle, and no one has yet identified HCV having escaped the requirement for miR-122 through resistance to miR-122 antagonists, we hypothesized that point mutations would not be tolerated within the miR-122 binding sites, but that we might isolate genomes having point mutations in non-essential sequences located between the miR-122 binding sites. We selected for escape mutations in cells

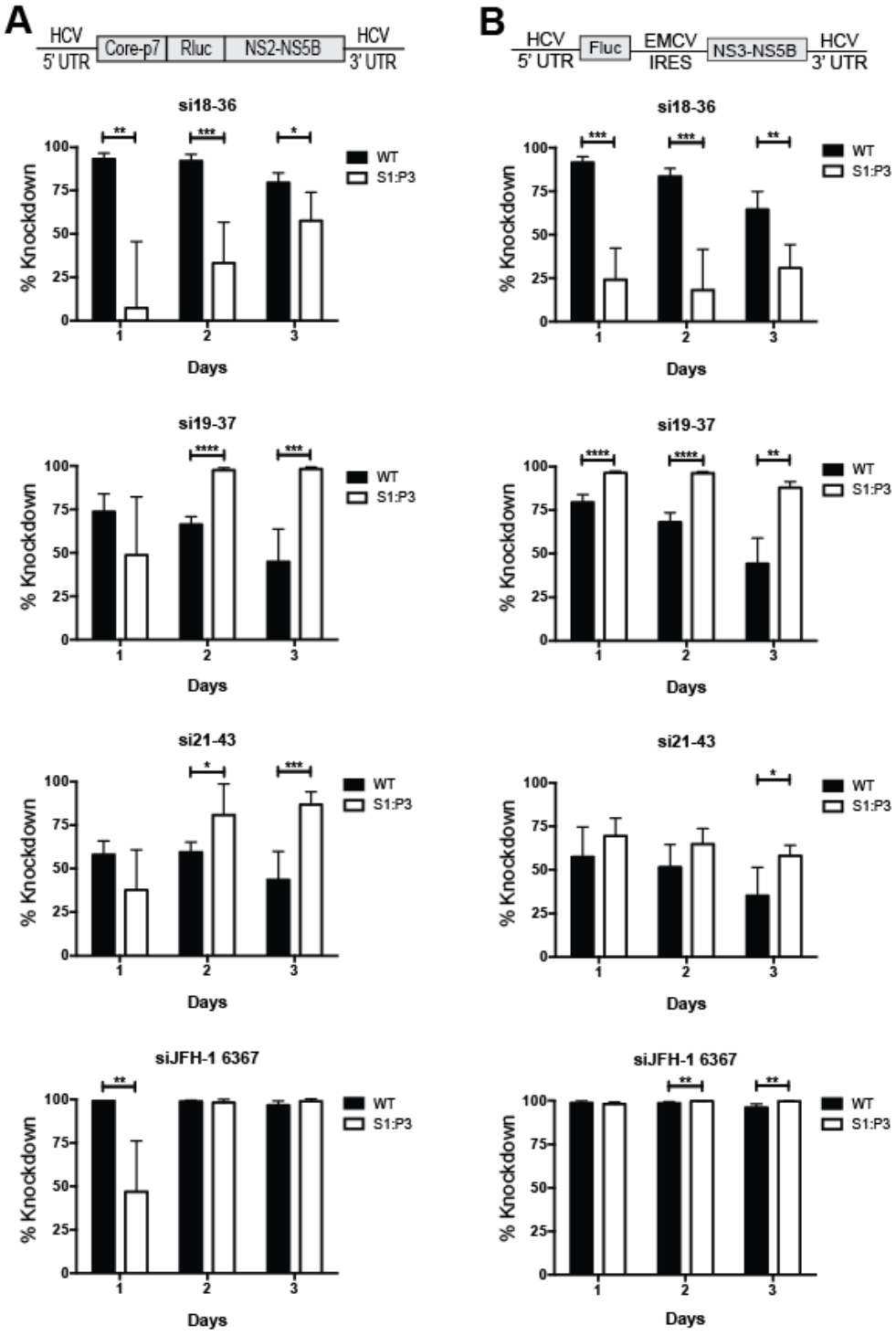


Figure 5.7 si18-37 and si21-43 knockdown of HCV S1:p3 is more potent than knockdown of WT virus. (A) The relative knockdown efficiency of the indicated siRNAs on J6/JFH-1 Rluc WT or S1:P3 mutant virus over a 3 day time course compared to siControl. (B) SGR JFH-1 Fluc was treated as described in (A).

harbouring bicistronic full-length and sub-genomic HCV RNAs since previous experiments demonstrated that sub-genomic replicons can replicate independently of miR-122 (albeit poorly) and thus might provide a background with a lower barrier to resistance in which to isolate mutants (116). After selection, we identified several point mutations located throughout the target sequence, including within miR-122 binding sites. These data indicate that HCV can replicate efficiently with a single intact miR-122 site. This is supported by our analysis of the p3 mutation in the context of the full-length HCV RNA and previous work showing that HCV RNAs having point mutations to miR-122 binding site 1 are viable (117). It is possible that the use of bicistronic constructs allowed the isolation of a more diverse set of mutants than would have been seen using a full-length RNA. Point mutations were present in all positions of the si18-36 target sequence that targeted the miR-122 binding region with the exception of the C at position 24 and suggests that this position may be the only non-variant position in this region of the genome. Interestingly, viruses having mutations outside of si18-36 target site, and within miR-122 binding site 2 were also selected. This suggests the possibility that the siRNA may have been able to bind to the second seed sequence to inhibit replication. Fewer point mutations arose from selection using si19-37. We believe this might be due to the fact that this siRNA spans both miR-122 sites and functions both as an siRNA and as a barrier to miR-122/Ago2 binding. It is also possible that some mutant viruses were eliminated due to poor replication, or reversion during the two weeks following siRNA treatment when cells were allowed to grow. The replicative fitness of the mutants will be explored in future experiments using full-length RNA, and analysis of the ability of full-length HCV to incorporate mutations in the miR-122 binding sites awaits future selection of full-length HCV RNAs expressing the neomycin resistance gene. In addition, we believe that it is likely that compensatory mutations elsewhere in the genome have arisen to influence the replicative fitness of the RNAs having mutations to the miR-122 binding sites. Sequencing full-length genomes to identify compensatory mutations could provide insight into the mechanism of action of miR-122.

The incorporation of a point mutation into the target site of a siRNA inhibits its knockdown efficiency. However, when the miR-122 binding region siRNAs were assayed for functionality against an HCV RNA having a point mutation within the target sequences for all three siRNAs, the effects on the siRNAs activity were variable. As one would expect, inhibition by si18-36 was attenuated by a point mutation in its target sequence but unexpectedly, inhibition by si19-36 and si21-43 was enhanced. The fact si18-36 loses effectiveness suggests that its main mechanism of inhibition was through siRNA cleavage. Given that the difference between si18-36 and si19-37 is a single nucleotide that extends into the second miR-122 binding site (Fig. 5.1A) we believe that si19-37 and si21-43 inhibit HCV replication by blocking miR-122 binding to site 2. Thus, we propose that si18-36, si19-37 and si21-43 inhibit WT HCV by binding to and cleaving the 5'UTR of the HCV genome. However, si19-37 and si21-43 also prevent miR-122 binding to both the first and second miR-122 binding sites. In the context of J6/JFH-1 Rluc S1:p3, si19-37 and si21-43 are more effective at inhibiting the virus, since replication of this RNA relies on miR-122 binding to site 2 when site 1 is mutated. This effectively blocks miR-122 from accessing HCV 5'UTR and blocks HCV replication.

That si19-37 may block both miR-122 binding sites would explain why fewer escape mutations were isolated compared to si18-36, since a greater complexity of mutations would be required by HCV to escape. Similarly, we expect that the virus would also have difficulty escaping from si21-43 and make these two siRNAs good candidates for future HCV therapies, or for use in conjunction with si18-36 to limit the replication of escape mutants.

6.0 LINKER BETWEEN CHAPTERS 5 AND 7

When we sequenced the 5'UTR of HCV RNA from cells that had undergone positive selection for seven rounds of treatment with an siRNA targeting the miR-122 binding region, we observed mutations in the miR-122 binding region as discussed in Chapter 5, but also in sequences downstream of the miR-122 binding region. These downstream mutations represent possible advantageous adaptation of the virus, and may also provide further insight into the role of miR-122 and HCV and thus are worthy of further study.

7.0 MULTIPLE ROUNDS OF TREATMENT WITH siRNA TARGETING THE miR-122 BINDING REGION OF HCV SELECTS FOR VIRAL GENOMES HAVING MUTATIONS THAT RESIDE OUTSIDE OF THE siRNA TARGET SEQUENCE AND miR-122 BINDING REGION

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7.1 Authors' contribution

Adam Huys performed all the experiments described and reported in the manuscript. Adam Huys wrote the manuscript, which was edited by Joyce A. Wilson

7.2 Abstract

Hepatitis C virus (HCV) requires the miRNA, miR-122 to bind to its 5'UTR for robust replication and translation. Due to this dependency, the miR-122 binding region of HCV is highly conserved across genotypes and has been demonstrated to be a target for efficient siRNA mediated degradation (Chapter 5). In the past, siRNA targeting of HCV has resulted in the selection of escape mutants having point mutations to the siRNA target sequences. Sequencing results presented in Chapter 5 indicate this is also true for siRNA targeting the miR-122 binding region. Further examination of the 5' UTR sequence from the selected HCV genomes demonstrated that not only do the selected viruses contain mutations in the siRNA target sequence but also mutations down stream of the siRNA target sequence. Several of these mutants occur in the AUG start codon of HCV and at conserved nucleotides predicted to have a modified confirmation upon miR-122 binding to the 5'UTR.

7.3 Introduction

Hepatitis C virus (HCV) is an enveloped virus with a positive sense RNA genome (18). The genome encodes 10 proteins, which are translated as a polyprotein from a single open reading frame. A cap-independent process initiates translation of the polyprotein by the recruitment of the 40S ribosome and limited initiation factors to a structured RNA element that forms an internal ribosome entry site (IRES) within the 5' untranslated region (UTR) of HCV RNA genome (55). The 5'UTR sequence is highly conserved across genotypes. Based on predicated secondary structure the HCV 5'UTR is divided into four domains. Domain I is required for replication, domains III and IV are required for translation, and domain II is required for both replication and translation. Together domains II, III and IV comprise the IRES.

The IRES coordinates initiation of translation. Domain III recruits the 40S ribosome and then all three domains (II-IV) assist in retaining and positioning the 40S ribosome in the proper orientation with respect to the AUG start codon, located in domain IV. Once the ribosome is properly positioned, domain III recruits eIF3, which associates with the 40S ribosome. The formation of the 40S ribosome-eIF3 complex allows the incorporation of the ternary initiation complex eIF2-GTP-$tRNA^{met}$. After, or during the formation of the 40S ribosome-eIF3-eIF2-GTP-$tRNA^{met}$ complex, eIF5 is included in the complex. The inclusion of all the factors in the complex leads to the phosphorylation of eIF5. Similar to its role in cap-dependent translation, phosphorylated eIF5 facilitates the recruitment of the 60S ribosome and the formation of the translational competent 80S ribosome (53-55). The coordinated recruitment of factors required for translation initiation by the highly structured HCV IRES emphasizes not only the importance of the secondary structure, but also the ability for the structures to interact with each other. The requirement for these highly sophisticated interactions is exemplified by conservation of 5'UTR sequences across genomes (117).

Domain I of HCV 5'UTR is not required for translation (55), but interacts with miR-122 and promotes both HCV replication and translation (104, 105, 108, 110).

Two miR-122 molecules, along with argonaute 2 have been shown to bind at two seed sites located within domain I (111, 114, 193). The interaction between miR-122 and HCV 5'UTR protects the viral genome from Xrn1 5'-3' exonuclease degradation, and leads to an increase in viral translation and replication (113). The increase in viral translation is the result of the increase in RNA abundance provided by miR-122 protection, but the increase in replication is not solely linked to the increase in RNA abundance through protection from Xrn1 (113). Another possibility is that miR-122 binding modifies the structure of the 5'UTR. Utilizing selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) selective analysis, two separate groups have reported that miR-122 binding to the 5'UTR induces downstream changes in the 5'UTR (115, 120). These changes could hypothetically increase or decrease HCV translation and replication.

We have used siRNAs that target the miR-122 binding region within the 5'UTR to select viral RNAs having point mutations to the miR-122 binding sites. We observed the selection of RNAs having mutations to the siRNA-targeted region (described in Chapter 5) and also RNAs having mutations down stream, in the conserved regions of the IRES. Their selection suggests that some of the mutations afford the virus a selective replication advantage when the miR-122 binding region is being targeted. We speculate that some of the mutations are general growth enhancing mutations, and that some may be compensatory mutations that allow the virus to escape dependence on miR-122. In this chapter we will describe the mutations we have selected and compare them with nucleotides whose conformations were affected by miR-122 binding. We will also propose future studies to investigate the impact of the mutations on HCV replication and its dependence on miR-122.

7.4 Materials and methods

7.4.1 Cell culture

Huh7.5 cells harboring J6/JFH-1 Neo Rluc were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 0.1nM non-essential amino acids (Wisent, Montreal, Canada), 100µg/ml Pen/Strep (Invitrogen, Burlingtion ON, Canada) and 800µg/ml G418 Sulfate (Wisent, Montreal, Canada) to maintain the HCV replicon. This stable cell line was provided by provided by Dr. Liu Qiang.

7.4.2 Small interfering RNAs (siRNA) sequence

The siRNAs that target the miR-122 binding region have been described previously in Chapter 5.3.3.

7.4.3 Electroporation of Huh7.5 cells harbouring J6/JFH-1 Neo Rluc

The electroporation of cells was conducted as described previously (116)

7.4.4 Escape mutant selection assay

The selection of escape mutants was performed as previously described in Chapter 5.3.8.

7.4.5 RNA purification

RNA was purified as previously described in Chapter 5.3.10.

7.4.6 Sequencing of the 5' portion of HCV

HCV was sequenced from the beginning of the neomycin gene to the 5'end as previously described in Chapter 5.3.11.

7.4.7 Analysis of sequencing data

All sequences were analyzed using Clone Manager software.

7.5 Results and discussion

To identify virus mutants that have escaped miR-122 binding sites targeting siRNA, Huh7.5 cells harbouring J6/JFH-1 Neo Rluc were subjected to seven rounds of treatment with individual siRNA targeting the miR-122 binding region of HCV, and selection for cells that had retained the HCV RNA (Fig. 7.1A, B and C). After the 7th round of treatment/selection nucleotides 17–409 of J6/JFH-1 Neo Rluc were sequenced and compared with the original J6/JFH-1 Neo Rluc sequence. From the selection we expected to identify HCV RNAs having mutations that mediate escape from the siRNA based on mutations to the miR-122 sites, and perhaps escape from the requirement for miR-122, and also mutations that simply enhance HCV replication efficiency. We also sequenced RNA from cells treated seven times with an siRNA targeting the NS5B coding region (siJFH-1 6367) and from cells that received no treatment, but were continuously passaged to identify HCV mutations that enhance replication in the presence of an siRNA that targets another part of the genome, or mutations that simply enhance HCV replication without siRNA knockdown pressure.

Sequencing results were obtained from 7 different experiments. For si18-36, RNA sequences from 3 independent experiments were analyzed. For si19-37, 2 independent experiments were sequenced, and for siJFH-1 6367, and no siRNA selection, clones from 1 experiment were sequenced. Sequencing data indicated that numerous mutations occurred throughout the 5'UTR in all the experiments

Figure 7.1

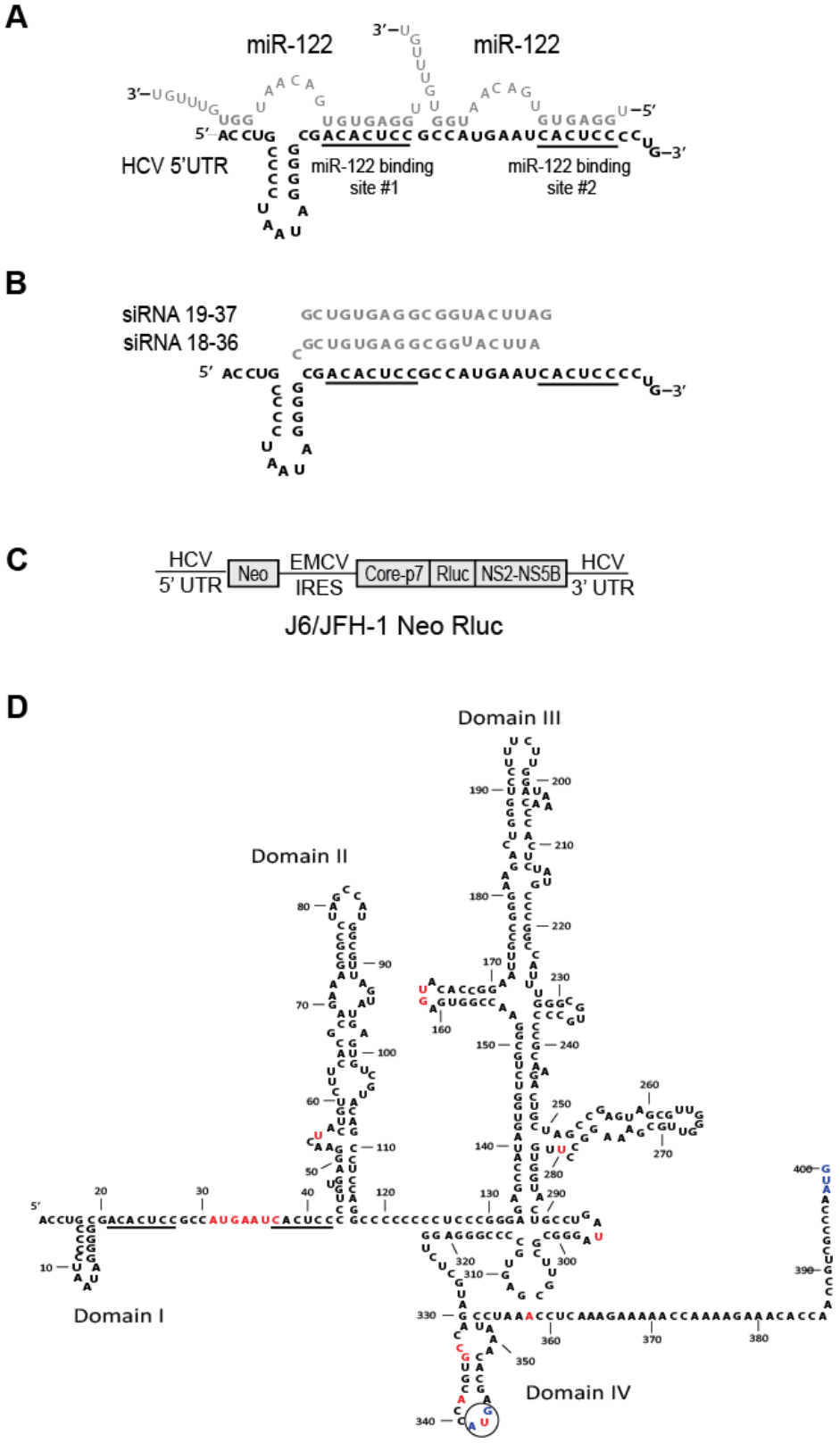

A
miR-122
miR-122
HCV 5'UTR
miR-122 binding site #1
miR-122 binding site #2
B
siRNA 19-37
siRNA 18-36
C
HCV 5' UTR
Neo
EMCV IRES
Core-p7
Rluc
NS2-NS5B
HCV 3' UTR
J6/JFH-1 Neo Rluc
D
Domain I
Domain II
Domain III
Domain IV

Figure 7.1 Binding of miR-122 and siRNA designed to target the miR-122 binding region to HCV 5'UTR. A schematic diagram depicting how miR-122 (A) and (B) siRNA targeting the miR-122 binding region interact with the 5'UTR of J6/JFH-1 Neo Rluc (C). The predicted secondary structure of HCV 5'UTR (D). The underlined nucleotides indicate where miR-122 binds and the red nucleotides indicate nucleotides which accessibility is predicted to change upon miR-122 binding. The circled AUG represents the initiation codon for core and the blue AUG indicates the beginning of the neomycin gene.

(Appendix 1). The differentiation of mutations between groups, which had been treated with siRNA targeting the miR-122 binding region, and the control groups were not visibly obvious with the exception of the siRNA target region which was discussed previously (Chapter 5).

For our analysis we focused on nucleotide mutations that correlated with those identified previously by SHAPE analysis to undergo a conformation change following miR-122 binding. Briefly, SHAPE assesses the accessibility of the ribose 2'hydroxyl group of RNA by adding a bulky adduct. Modified, and thus accessible nucleotides are then detected based on the ability of the bulky adduct to prevent RNA extension by reverse transcriptase. The availability of the 2'hydroxyl group varies depending on the structural context of each nucleotide and the interactions it is involved in (119). Thus, through SHAPE analysis the availability of a nucleotide can be determined and used to assess RNA conformation and conformational changes. Two groups, Pang *et al.* and Mortimer and Doudna, used SHAPE analysis to assess changes in HCV 5'UTR nucleotides availability with and without miR-122 binding (115, 120). Both studies showed reduced accessibility of nucleotides at the miR-122 binding sites following miR-122 annealing, as would be expected following RNA-RNA annealing. Pang *et al.* also found nucleotides outside of the miR-122 binding site that were modified by miR-122 binding, but attributed their modification to a direct interaction between 3' nucleotides of the miR-122 (not the seed binding region), and the HCV genome. Mortimer and Doudna identified 10 nucleotides outside of the miR-122 binding region in which nucleotide accessibility changed upon binding of miR-122 (Fig. 7.1D). 5 nucleotides became less accessible (nucleotides 163, 280, 296, 338 and 343) and 5 became more accessible (nucleotides 55, 162, 333, 334 and 358). We speculate that perhaps some of the mutations we observed in our sequencing data would match those reported by the two groups of researchers to be modified by miR-122 binding (115, 120). Interestingly, we identified in our escape mutant population, mutants having changes to 5 out of the 10 nucleotides identified by Mortimer and Doudna to be modified by miR-122 binding (Fig 7.2A). In addition, in control passaged cells we identified 1 clone with a mutation to nucleotide 358 (out of 3 total mutants

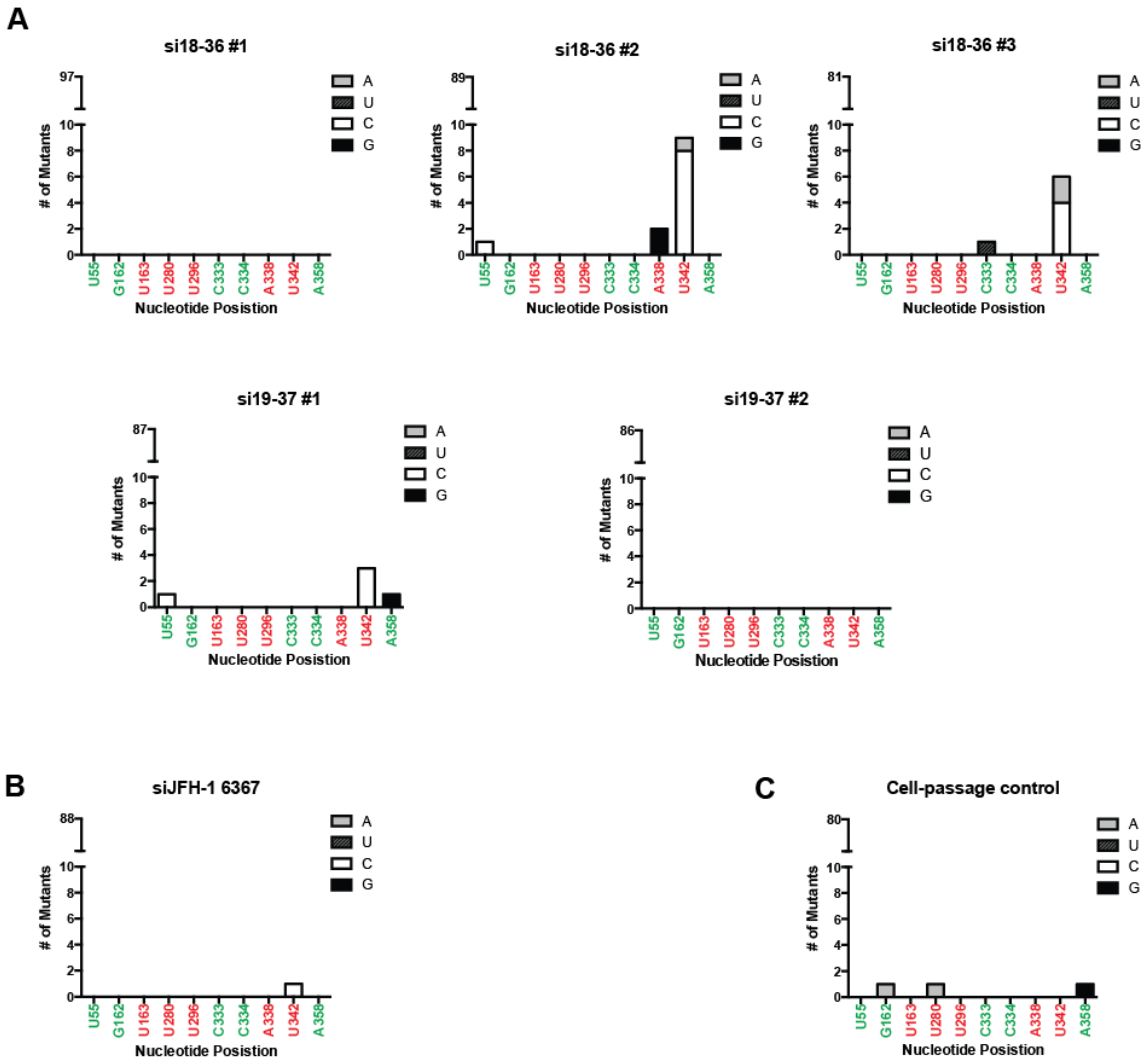


Figure 7.2 Point mutations at nucleotides predicted to be more accessible when miR-122 is bound to the 5'UTR. (A) The numbers and locations of J6/JFH-1 Neo RLuc sequences retrieved in cells treated with siRNA targeting the miR-122 binding region having a point mutation at one of the 10 nucleotides outside of the miR-122 binding region that were predicted to have differed accessibility when miR-122 is bound (B) siJFH-1 6367 and (C) cell-passage control. The nucleotides predicted to have increase accessibility are coloured green while the nucleotides predicted to be less accessible are coloured red.

identified) which also overlapped those identified by Mortimer and Doudna (Fig. 7.2C). We speculate that these mutations may mediate enhanced HCV replication, and that some may enhance HCV replication in the absence of miR-122, but roles for these mutations in enhancing HCV replication, or enhancing replication independent from miR-122 remain to be confirmed experimentally.

In spite of our current lack of replication fitness data we can discuss the nature of the selected mutants, shown in (Fig. 7.3) and speculate on structural changes they might induce. The first mutation was at position 55. The SHAPE availability at position 55 is increased with miR-122 binding suggesting that the RNA conformation at this site might be modified by miR-122 (115). In our selection assay this position was mutated from U to C. In WT virus sequence this nucleotide is located in a bulge region in stem-loop (SL) II and is not predicted to base pair with other nucleotides in the structure of the IRES (120). The effect this mutation has on HCV is unknown and no available G appears to be readily available to bind with the mutant C. The second mutation at a site predicted to be modified by miR-122 was at position 333. SHAPE data indicates that miR-122 binding increases SHAPE accessibility of this site (115) and our sequence data identified 1 mutant that had the C mutated to U at this position. In the WT sequence this nucleotide is predicted to be unpaired as part of a bulge in SL-IV, and was found be more SHAPE reactive when miR-122 is bound (115). Interestingly, the mutation of this site from C to U would allow base pairing with an A at position 349 which may add stability to SL-IV. The third interesting mutation, an A to a G mutation at position 338, was isolated in two mutants. In WT virus this nucleotide is unpaired and within the loop of SL-IV, and miR-122 binding reduced SHAPE reactivity (115). The mutation from A to G does not induce any obvious change in base pairing across the predicted loop. Finally, we identified an RNA carrying an A to G mutation at position 358 in cells treated with miR-122-binding site siRNAs. This region is downstream of the HCV AUG start codon but is predicted to be part of the IRES. It is predicted to be unpaired in the WT HCV genome, and becomes more SHAPE reactive with miR-122 binding (115). As we also isolated a mutant in our cell-passage control containing the same point mutation, the advantage of the mutation is unknown and may not

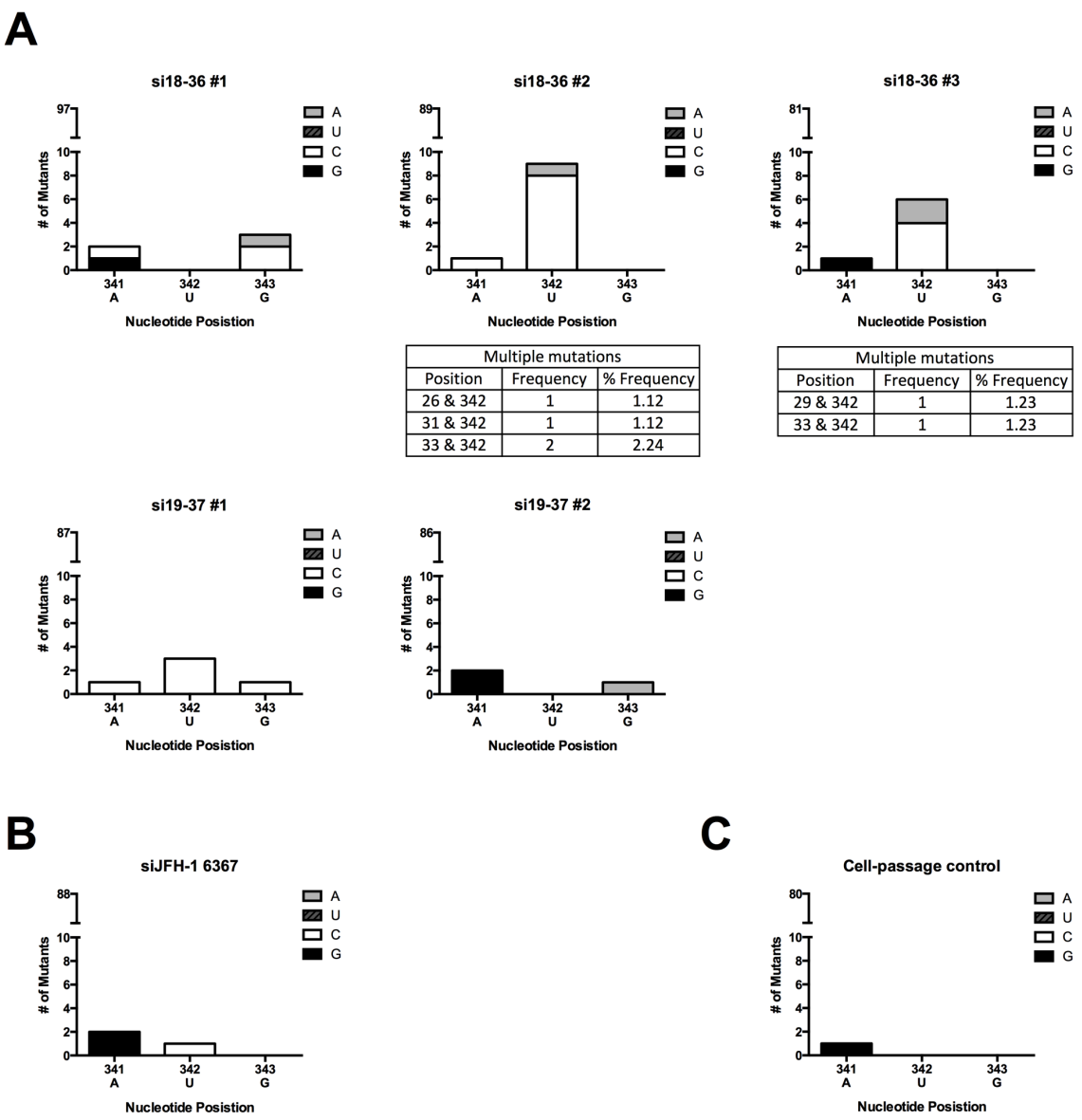
Figure 7.3

Clone #	Base	WT base	Accesibility after miR-122 binding	Mutation base	Other point mutations in the clone
1	55	U	+	C	72 (G)
2					-
3	333	C	+	U	-
4	338	A	-	G	204 (C)
5					-
6	342	U	-	C	224 (C), 355 (C)
7					-
8					31 (U)
9					26 (U)
10					263 (A)
11					-
12					33 (A)
13					33 (A)
14					381 (G)
15					29 (A)
16					Insertion of a C between 117 - 118
17					381 (G)
18					161 (G), 215 (A), 249 (G)
19					191 (U), 384 (Δ), 386 (G)
20					93 (U), 383 (G)
21				A	-
22					33 (A), 224 (G), 326 (U), 380 (G)
23					381 (G)
24	358	A	+	G	-

Figure 7.3 Isolated mutations within the 5'UTR and their predicted interaction within secondary structure. A schematic diagram of the 5'UTR with the 5 nucleotides predicted to have different availability upon miR-122 binding indicated by arrows. A table demonstrating the isolated clones, the mutation that arose at the nucleotide predicted to have a different availability upon miR-122 binding and other point mutations found within the individual clone.

be related to miR-122 binding of the 5'UTR. In general, escape mutants having mutations to nucleotides shown to be modified by miR-122 may be adaptive mutations that enhance replication in general. They may also specifically enhance HCV replication in the absence of miR-122 binding, but this is less likely since they do not reside on RNAs that also have mutation to the miR-122 binding sites, however, this remains to be confirmed experimentally. In the future these mutations should be tested in the context of full-length and sub-genomic HCV model replication systems to determine their influence on HCV replication and on its dependence on miR-122.

Mutants to one other nucleotide whose SHAPE accessibility was found by Mortimer and Doudna to be modified by miR-122, nucleotide 342, was also isolated multiple times in our siRNA escape mutants. We isolated 18 mutants at position 342 in RNA from the cells that were treated with siRNAs targeting the miR-122 binding region (Fig. 7.2A and Fig. 7.3), and interestingly, it was identified in many clones that also had mutations to the miR-122 binding sites (Fig 7.3: table). A mutation at position 342 was also identified in one clone from cells treated with siJFH-1 6367 (Fig. 7.2B) suggesting that it may have a general positive impact on replication efficiency. This nucleotide resides within the loop of stem-loop IV and is predicted to be less accessible following miR-122 binding. In WT viral RNA this nucleotide is the U of the AUG start codon of the HCV polyprotein, but in our HCV RNA constructs this start codon initiates expression of the neomycin selection gene (Fig. 7.1C) but not the HCV proteins. It is likely that HCV RNA replication was enhanced by abolishing translation of the selection gene because this would reduce cellular resources required, but how these replicons, which no longer express the neomycin resistance gene were retained following G418 selection is unknown. Interestingly, mutations within the start codon were not limited to position 342 in RNAs isolated from cells treated with miR-122 binding siRNAs and siJFH-1 6367. There were also numerous mutations at both position 341 and 343, the A and G of the AUG start codon (Fig. 7.4A and B). One possibility is that translation initiation in the mutant genomes used alternative start codons. ACG or AAG, or an alternative method of translation initiation. Alternatively, a downstream start codon may



Multiple mutations		
Position	Frequency	% Frequency
26 & 342	1	1.12
31 & 342	1	1.12
33 & 342	2	2.24

Multiple mutations		
Position	Frequency	% Frequency
29 & 342	1	1.23
33 & 342	1	1.23

Figure 7.4 siRNA targeting the miR-122 binding region induce point mutations in the HCV start codon. (A) The numbers of J6/JFH-1 Neo RLuc sequences retrieved having a point mutation in the start codon after treatment with an siRNA targeting the miR-122 binding region, (B) siJFH-1 6367 or (C) cell-passage control.

have been used to express the neomycin resistance gene, but since the HCV IRES does not allow ribosome scanning this is unlikely (271-275). Also, replicons may have been selected in cells having gained an alternative method to resist G418 selection, or harbouring a second replicon expressing the resistance gene and perhaps virus replicate better when the 5' end of the virus in not involved in translation and thus more available for replication. Since 6 of these mutants had a second mutation in the miR-122 binding site it suggests that it may be a compensatory mutation that promotes RNA replication in the absence of miR-122 binding. These observations were depended on the use of a bicistronic replicon to allow for mutations that may abolish IRES translation and may not be viable in the context of a WT virus, although this remains to be determined. However, we believe that this array of escape mutations could provide insight into the role of miR-122.

8.0 GENERAL DISCUSSION AND CONCLUSIONS

8.1 General discussion

This thesis work began in an environment were an interaction between HCV, miR-122 and components of the miRNA suppression pathway had only recently been observed and reported (104, 105, 136). The field was in the midst of an explosion of exploration in an attempt to better understand the link between HCV, miR-122 and the miRNA suppression pathway. In the years since, our understanding of how HCV interacts with miR-122 and the miRNA suppression pathway has grown considerably and the work presented in this thesis has contributed to the field.

Our research conducted on the DEAD-box helicase DDX6 has demonstrated that it is required for optimum HCV replication and translation. We hypothesized that the effect DDX6 had on augmenting HCV replication was linked to HCV's dependency on miR-122, as DDX6 was reported to augment miRNA suppression through an interaction with Ago1 and Ago2, two proteins that aid in miR-122 interaction with HCV (153). Contrary to our hypothesis, we demonstrated that DDX6's effect on HCV replication and translation is independent of miR-122. As these results were unexpected, and given the observations of others that DDX6 affects the miRNA suppression pathway, we examined whether DDX6 silencing impeded the miRNA suppression pathway in Huh7.5 cells. Our findings indicate that in the context of Huh7.5 cells DDX6 had no effect on miRNA suppression. We confirmed this observation in the context of miR-122 suppression, and also using a system to measure suppression by miCXCR4, the identical assay used in the previous study conducted in HeLa cells (153). This suggests that DDX6 does not augment miRNA targeted gene suppression, at least in Huh7.5 cells. However, we cannot rule out the possibility that Huh7.5 cells may have a secondary mechanism that is not present in HeLa cells that renders DDX6 activity in miRNA suppression redundant.

When miR-122's interaction with HCV was first reported, it was suggested to only augment replication and have no effect on HCV translation. However, contradictory reports were soon published demonstrating that miR-122 could augment HCV translation as well as replication (104, 105, 108, 110-112). Our observations not only strongly support that miR-122 promotes HCV replication but support its role in stimulating translation.

While our data indicates that DDX6 promotes HCV replication, our hypothesis that the mechanism by which DDX6 augments HCV via miR-122 was not supported. This left us to re-hypothesize a mechanism by which DDX6 augments HCV replication. Since DDX6 is required for p-body formation and maintenance, a possible mechanism by which DDX6 supports HCV replication could be related to p-body formation. One possibility is that p-bodies are important for HCV replication. Other research groups have demonstrated that p-body structures are not required for HCV replication, and similarly to the flaviviruses, WNV and Dengue virus, HCV infection leads to a decrease in visible p-body structures over time (139, 157, 159, 257, 258). However, we cannot rule out the possibility that smaller p-body aggregates, which are not microscopically visible, might function to promote HCV replication. Interestingly as the presence of visible p-bodies decrease during HCV infection, DDX6 co-localizes with other p-body resident proteins at sites of HCV replication which, suggests that multiple p-body proteins may facilitate the HCV life cycle (258), but their mechanism of action remains unknown.

Since DDX6 is an RNA helicase it is also plausible that DDX6 directly interacts with the HCV genome, perhaps to unwind the RNA during translation or replication. DDX6 associates with the HCV genome, but since the interaction requires HCV core protein, and core protein is not required for the impact of DDX6 on HCV translation and replication, the role of DDX6-core-genome interaction is unknown (259). One possible explanation for the interaction between DDX6, core and the viral genome could be that DDX6 is also required for HCV assembly. Although DDX6 silencing results in a decrease in infectious particles released it will be difficult to determine if the decrease is correlated with its impact on HCV replication, translation, or assembly since it is difficult to design experiments that assess HCV RNA replication

and translation separate from virion assembly. In addition, DDX6 and other p-body proteins that co-localize with core at the site of HCV replication and assembly may also suggest a role for DDX6 and other p-body proteins in HCV virion assembly (258). Interestingly, DDX6 has a role in the assembly of other viruses such as HIV and PFV (196, 200).

Even if DDX6 does assists in HCV assembly, our translation and replication assays suggest an additional role in HCV replication and translation. It is currently unknown if the influence of DDX6 on HCV translation is responsible for its apparent impact on augmentation in replication, or if DDX6 augments both HCV translation and replication, perhaps through separate mechanisms. This question will remained unanswered until such time as experimental systems are designed that allow the separation of HCV translation from replication. Evidence suggests that DDX6 does not function by binding directly to HCV genomic RNA since RNA was not immunoprecipitated with DDX6 (259).

Interestingly, simultaneous to our research it was reported that DDX6 is recruited to WNV and Dengue virus replication sites (158, 159). DDX6 knockdown was also reported to lead to a decrease in Dengue virion release (158). However, whether DDX6 silencing leads to a decrease in RNA replication or translation has yet to be reported in the case of WNV or Dengue virus, although DDX6 has been demonstrated to physically interact with the 3'UTR of DENV-2 (158). Similarly to HCV, during the course of infection with WNV and Dengue virus p-bodies disappear (157, 258). These similar observations between HCV, WNV and Dengue virus suggest that they may be utilizing DDX6 in a similar manner for replication, translation, and/or assembly. I believe these observations also support our observations that the effect of DDX6 on HCV is independent of miR-122 since WNV and Dengue virus have not been demonstrated to utilize a miRNA for replication.

Because of the rapid progression of research on the role of p-bodies in the HCV life cycle, and our desire to focus our research on the mechanism of action of miR-122, we re-focused our research to the study of a mechanism to inhibit HCV by exploiting its dependence of on miR-122.

Given HCV needs to bind miR-122 and the conserved nature of the miR-122 binding region across genotypes, we hypothesized that the miR-122 binding region would be a good target for siRNA mediated HCV RNA inhibition as a possible treatment. Our goals for these experiments were two fold. Firstly, we hypothesized that the siRNA could be used to knockdown HCV RNA levels in infected cells, but secondly, given the ability for HCV to escape siRNA treatment through incorporation of point mutations to the siRNA target sequence, we thought the siRNA could be used as a novel mutagenic tool to identify the mutational flexibility of the miR-122 binding region. These mutations could be further analyzed to gain a better understanding of the relationship between HCV and miR-122. As expected our results indicated that the miR-122 binding region was susceptible to siRNA-targeted degradation. The three siRNAs, designated si18-36, si19-37 and si21-43, designed to target the miR-122 binding region were able to cause a potent decrease in HCV replication levels in cell culture. This was observed in J6/JFH-1 Rluc, a full-length construct capable of fulfilling the entire viral life cycle, and a sub-genomic replicon capable of replicating but not assembling viral particles. Although all 3 siRNAs inhibited HCV replication, they varied in effectiveness with si18-36 being the most effective followed by si19-37 and si21-43 respectively.

To evaluate the ability of HCV to escape the siRNAs targeting the miR-122 binding region we used HCV constructs that encoded a neomycin resistance gene. The neomycin resistance gene allowed us to positively select for cells containing replicons that were not inhibited by the siRNA. After seven rounds of treatment, HCV knockdown assays did not suggest the emergence of escape mutants to the miR-122 binding-site targeting siRNAs, but they were detected in the positive control group (siJFH-1 6367). These results suggested that siRNAs targeting the miR-122 binding region could be used as potential treatment for patients infected with HCV. However, when we sequenced the 5'UTR region of the selected RNAs we observed many genomes having point mutations in the siRNA targeted regions. This suggested that HCV RNAs could escape siRNA knockdown by introducing mutations into the miR-122 binding sites and the surrounding sequence. To determine whether HCV RNAs having a point mutation within the siRNA target site had

escaped siRNA inhibition, we examined knockdown effectiveness of the siRNAs against a known replication competent HCV RNA having a point mutation within the miR-122 binding region. This HCV construct (HCV S1:p3) contains a point mutation in the first miR-122 binding site and replicates about 10 fold less efficient than WT virus constructs. The S1:p3 mutation falls within the sequence targeted by all 3 siRNAs designed to target the miR-122 binding region. We expected that the sequence mismatch between HCV S1:p3 and the siRNAs would attenuate or abolish siRNA knockdown ability, and this is what we observed for si18-36, but the inhibitory effects of si19-36 and si21-43 were unexpectedly enhanced on the mutant virus. Specifically, si18-36, which is the most effective against WT virus, lost most of its potency. However, si19-36 and si21-43 which, were less effective than si18-36 at inhibiting WT virus were more effective on the mutant virus. This observation was surprising, as we had predicted the point mutation would render all 3 siRNAs less potent, since the imperfect complimentary of the siRNA with its target would prevent siRNA-mediated cleavage.

A possible explanation for this observation is that si19-37 and si21-43 in association with Ago2 are a competitive inhibitor that blocks miR-122/Ago2 complexes from binding to miR-122 binding site 2. The location of the point mutation in the HCV S1:p3 construct prevents miR-122 from binding to the first miR-122 seed sequence in the 5'UTR, therefore replication of this construct relies on miR-122 binding to the second site. Since si19-37 and si21-43 overlap the second miR-122 binding site and si18-36 does not, I suspect that these siRNAs retain knockdown efficacy by blocking miR-122 binding to site 2.

As the administered siRNAs are double stranded, the efficiency by which they are processed and incorporated into Ago2/RISC complex will also affect inhibition efficiency. As si18-36 and si19-37 were demonstrated to function equally well as a siRNA when suppressing mRNA expression in a control assay, we conclude that they are processed and incorporated into a RISC with similar efficiency. Therefore it is reasonable to expect the difference in their function against HCV is not a result of their ability to be incorporated into the RISC. On the other hand, si21-43 was observed to be less effective at suppressing a control mRNA than si18-36 and si19-

37 and we concluded that it was less functional against WT HCV because it is not processed into a RISC as efficiently.

We propose that si18-36 is more effective at inhibiting WT HCV than si19-37 because of differences in how they physically interact with the 5'UTR. The difference between the two siRNA is 1 nucleotide and we believe that it is significant that the one different nucleotide targets the first nucleotide of the second miR-122 seed sequence on HCV 5'UTR. Thus, si18-36 targets only the first miR-122 binding site and the space between the two seed sequences, while si19-37 targets the first miR-122 seed sequence, the space between, and 1 nucleotide of the second miR-122 seed sequence. Therefore we speculate that si18-36 is competing with just one miR-122 for binding to the 5'UTR, while si19-37 is less efficient since it is competing with two miR-122s for the 5'UTR. In the case of WT, we assume that the siRNA interaction with the 5'UTR leads to a cleavage of the genome, which prevents replication. In the case of the mutant construct, we speculate that si18-36 is ineffective since it does not induce cleavage due to the sequence mismatch, and does not block miR-122 binding to the second seed site, and that si19-37 is effective since it competitively inhibits miR-122 binding to site 2. This model also explains why si21-43 is more potent against the mutant HCV construct than the WT, since it is predicted to also compete with miR-122 for binding the second miR-122 seed sequence. This observation suggests that siRNA targeting the miR-122 binding region has a potential dual mechanism of inhibiting HCV replication: first by the classical siRNA mediated target cleavage and second by acting as a competitive inhibitor for the miR-122 binding sequences.

Currently a promising treatment for HCV is utilizing LNA-122 to bind and sequester miR-122, thus preventing it from being used by HCV. It is possible that combination treatment of LNA-122 with siRNA targeting the miR-122 binding region could be an even more efficient treatment. The combination treatment would have three modes of action, miR-122 sequestration, HCV RNA cleavage, and by blocking any residual miR-122 from binding to the 5' UTR. The combination treatment may also function synergistically, since firstly, siRNA knockdown may enhance degradation of HCV genomes to which miR-122 does not bind, secondly,

sequestration of miR-122 may enhance siRNA cleavage of the naked 5'UTR, and thirdly, the siRNA could out compete any residual miR-122 that LNA-122 did not sequester. Synergism between LNA-122 and siRNAs targeting the miR-122 sites is currently being assessed.

Another surprising outcome from these studies was that in si18-36 treated cells escape mutants were viable. Viral RNAs isolated and sequenced after seven rounds of treatment with si18-36 and selection with G418 had mutations throughout the 5'UTR including the miR-122 binding region and siRNA target sequence. The 5'UTR of the si19-37 treated cells also contained mutations throughout. With further analysis we concluded that the potency of si18-36 against a miR-122 binding site 1 point mutant S1:p3 was substantially decreased compared to WT explaining why viruses having point mutations were selected.

Each mutation observed within the miR-122 binding region represents a possible escape mutant and will be cloned into J6/JFH-1 Rluc and assayed for its effect on replication and its ability to replicate in the presence or absence of the siRNA and miR-122. We observed several mutations downstream of the miR-122 binding region whose confirmation were found previously by SHAPE analysis to be modified by miR-122 binding to the 5'UTR (115). These mutations will also be cloned into J6/JFH-1 Rluc and assayed for their functionality in the presence or absence of miR-122. Of note, we observed several mutations in HCV initiation codon, suggesting that some escape mutants had adaptive mutations in which reporter gene translation may have been abolished. Evidence suggested that these mutants enhanced RNA replication independent from an effect on miR-122 activity since they were also isolated in a control experiment to select for siRNA escape mutants against a siRNA that did not target the miR-122 binding sites. These mutations are certainly due to the bicistronic construct used, since if these mutations abolish virus polyprotein translation in a full-length monocistronic construct, replication would also be abolished. Thus, we also plan to isolate escape mutants in the context of full-length monocistronic HCV RNAs in the future.

Utilizing siRNAs targeting the miR-122 binding region we have achieved both of our objectives. Firstly, we have shown a proof of principle experiment that they

can be used as a possible treatment for HCV, and surprisingly, their inhibition of HCV is potentially though two mechanisms. Secondly, the siRNAs induced potential escape mutants with unique sequences, which upon further analysis will demonstrate the mutational flexibility of the miR-122 binding region that could lead to the evolution of a virus that can grow independent from miR-122 which would be a valuable tool to study the mechanism of action of miR-122.

8.2 General Conclusions

- DDX6 augments HCV translation.
- DDX6 augments HCV replication.
- The mechanism of DDX6 augmentation of HCV translation and replication is separate from that of miR-122 augmentation of HCV translation and replication.
- DDX6 does not promote miRNA directed gene silencing in Huh7.5 cells.
- miR-122 augments HCV translation.
- siRNAs designed to target the miR-122-binding region inhibit HCV replication.
- The siRNA targeting the miR-122 binding region have variable inhibitory effects on viral RNA having point mutations within the target sequences which suggests that they may inhibit HCV replication by two mechanisms.
- Multiple treatments of an HCV replicon with siRNA targeting the miR-122 binding region selects for mutations within the miR-122 binding region, siRNA target sequence, HCV IRES, and HCV initiation codon.

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10.0 APPENDIX 1

586 5'UTRs of HCV were sequenced from cells that were positively selected to retain the HCV replicon J6/JFH-1 Neo RLuc, after they had been treated with si18-36, si19-37, or siJFH-6367 seven times. An additional 96 5'UTRs of HCV were sequenced from cells harbouring J6/JFH-1 Neo RLuc, which had been passaged continuously in the presence of G418 while the siRNA treatments were being administered. The nucleotides coloured red and green in the "mutation site" column represent the location of the first and second miR-122 binding sites, respectively. The boxes in the "mutation site" and "WT nucleotide" column coloured green indicate the HCV start codon while the ones coloured blue represent the neomycin start codon. The boxes after the HCV start codon alternate every 3 nucleotides to represent the amino acid codons. The numbers in the experiment columns represent the designated clone and its colour indicates the mutation in the "mutation description" column.

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation discription	WT nucleotide
	Clones (97/106) 23 WT	Clones (89/96) 18 WT	Clones (81/96) 16 WT	Clones ((87/96) 25 WT)	Clones (86/96) 13 WT	Clones (88/96) 38 WT	Clones (80/96) 28 WT		
WT	3, 4, 5, 11, 24, 25, 27, 28, 29, 30, 34, 35, 36, 45, 46, 48, 50, 51, 66, 73, 76, 85, 88	11, 25, 35, 37, 43, 46, 52, 57, 64, 67, 71, 77, 79, 86, 87, 88, 91, 95	4, 10, 11, 24, 25, 32, 35, 36, 40, 41, 42, 55, 66, 75, 87, 89	5, 9, 11, 12, 15, 24, 26, 28, 30, 31, 32, 40, 41, 43, 47, 48, 49, 50, 51, 53, 59, 62, 79, 82, 90	3, 15, 18, 21, 26, 36, 46, 50, 54, 64, 73, 75, 93	2, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 26, 27, 30, 31, 34, 35, 36, 37, 38, 40, 44, 46, 48, 49, 53, 55, 56, 60, 61, 62, 63, 65, 71, 73, 79, 85, 95	4, 10, 16, 23, 27, 29, 32, 34, 35, 39, 40, 46, 48, 52, 53, 54, 56, 62, 63, 65, 67, 68, 75, 79, 80, 88, 91, 96		
17					13			G to A	G
18					13			G to A	G
19									C
20		9						G to A	G
21									A
22	22							C to A	C
23		28			65			A to G, A to C	A
24									C
25		12, 28				67, 78		T to C	T
26	67	30, 32						C to A, C to T	C
27	20	90						C to G, C to A	C
28	14, 22, 38,	1, 13, 44, 47, 92		92	7, 28, 31, 47, 91, 94			G to A, G to T, G to C	G
29	57		15	19				C to A, ,C to T	C
30	98			58	51, 53, 66			C to G, C to T	C
31	13, 41, 82	19, 27	23, 94					A to C, A to T	A
32									T
33		3, 15, 16, 22, 68, 69, 73, 78, 81	63, 68, 69, 80					G to C, G to A	G
34	49, 63	38, 72	44	17, 58	47, 51, 53, 66			A to C, A to G	A
35	78	19		8				A to G	A
36	94, 98							T to A	T
37	94							C to A	C
38									A
39	97							C to A	C
40									T
41	59, 97							C to A	C
42	97							C to A	C
43									C
44									C
45									T
46									G
47									T
48									G
49									A
50									G

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation description	WT nucleotide
51									G
52									A
53									A
54	93							C to T	C
55		63		73				T to C	T
56									A
57									C
58									T
59									G
60									T
61									C
62		21, 40, 73, 74				57, 82		T to C	T
63									T
64									C
65									A
66									C
67									G
68									C
69		26						A to G	A
70									G
71	26, 98		60	96	16			A to G	A
72		63		38	38	64	18	A to G	A
73									A
74									G
75									C
76						64		G to A	G
77									C
78							72	C to T	C
79									T
80									A
81									G
82									C
83									C
84									A
85							24	T to C	T
86									G
87									G
88									C
89									G
90						64		T to C	T
91									T
92			79					A to G	A
93				39, 61, 76, 89, 94				G to T	G
94									T
95									A
96									T
97									G
98									A
99		82						G to T	G

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation description	WT nucleotide
100									T
101									G
102									T
103							49	C to T	C
104			13					G to A	G
105									T
106	1, 86		12, 37	81		25		A to G	A
107									C
108			53					A to G	A
109									G
110									C
111									C
112									T
113									C
114							92	C to T	C
115									A
116									G
117									G
118									C
119									C
120									C
121							74	C to T	C
122									C
123									C
124									C
125									C
126									T
127									C
128									C
129									C
130									G
131									G
132									G
133									A
134									G
135									A
136		21						G to A	G
137									C
138									C
139						90		A to T	A
140									T
141						14		A to G	A
142									G
143									T
144									G
145									G
146					70		3, 82	T to C	T
147									C
148									T

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation description	WT nucleotide
149									G
150			53					C to T	C
151									G
152									G
153									A
154									A
155									C
156									C
157	75							G to A	G
158					34			G to A	G
159									T
160									G
161		51		25, 88			69	A to G, A to T, A to C	A
162							59	G to A	G
163									T
164			39	87				A to T, A to G	A
165									C
166	102, 103							A to G	A
167				1				C to A	C
168									C
169									G
170									G
171						8		A to G	A
172		94				72		A to G	A
173									T
174		55						T to C	T
175									G
176		19	7	86, 93				C to T	C
177									C
178									G
179									G
180									G
181		47			67	3	71	A to G	A
182			48, 79					A to C, A to G	A
183					27			G to A	G
184				64				A to G	A
185									C
186									T
187							92	G to A	G
188									G
189									G
190							1, 51	T to A	T
191				78				C to T	C
192									C
193	32	24				43	70, 72	T to A, T to C	T
194		3		88		59	17	T to C	T

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation description	WT nucleotide
195									T
196				10				C to T	C
197			14						T
198					80	47		T to C	T
199									G
200									G
201			53, 71		89			A to G	A
202		1, 41, 48, 53, 55, 60, 70						T to C	T
203			70					A to G	A
204		39		54				A to C, A to G	A
205				13				A to G	A
206									C
207							18	C to T	C
208							18	C to T	C
209						52		A to G	A
210									C
211									T
212	93							C to G	C
213			53	21, 45, 64, 67, 85				T to C	T
214					70			A to G	A
215				17, 25, 60	1, 4, 5, 7, 9, 12, 16, 17, 23, 31, 32, 33, 39, 40, 45, 47, 48, 59, 60, 61, 62, 68, 72, 79, 85, 89, 91, 92, 96	11, 58, 76	95	T to C, T to A	T
216			57					G to A	G
217	2					77, 92		C to T	C
218									C
219									C
220									G
221		65						G to A	G
222									C
223									C
224	17, 47, 57, 65, 80, 81, 94	5, 51	63	2, 46, 52, 57, 74	11, 55, 56, 88	21, 51	76, 90	A to G, A to C, A to T	A
225									T
226							36	T to C	T
227						41		T to C	T
228									G
229			14					G to A	G
230									G
231									C
232									G
233			52, 60			33, 47	83	T to C, T to A	T
234									G
235		26						C to T	C
236									C
237									C

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation description	WT nucleotide
239									C
240	78	58			43			G to A	G
241									C
242			93					A to C	A
243									A
244									G
245	12			66				A to G, A to C	A
246									C
247	85							T to C	T
248					23			G to A	G
249									C
250	106							T to C	T
251									A
252									G
253		12		70	1, 4, 5, 7, 9, 12, 16, 17, 31, 32, 33, 45, 47, 48, 59, 61, 62, 68, 72, 85, 89, 91, 96		33, 93, 94	C to T	C
254	64	10, 70						C to T	C
255									G
256									A
257									G
258									T
259									A
260	103							G to A	G
261						89		C to T	C
262									G
263		36	52	20		59	37	T to A, T to C	T
264	64		81					T to A, T to C	T
265									G
266									G
267	103							G to T	G
268						42		T to A	T
269							93	T to C	T
270									G
271									C
272									G
273									A
274									A
275						59		A to G	A
276									G
277									G
278									C
279									C
280							89	T to A	T
281									T
282									G
283									T

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation description	WT nucleotide
284									G
285									G
286	68						21, 26	T to C	T
287							9	A to G	A
288									C
289									T
290									G
291		60						C to T	C
292									C
293		54, 89					49, 69, 73	T to C	T
294									G
295									A
296									T
297									A
298									G
299									G
300									G
301									C
302									G
303									C
304									T
305									T
306									G
307									C
308			93					G to C	G
309						33		A to G	A
310									G
311							45	T to C	T
312									G
313									C
314									C
315									C
316									C
317									G
318									G
319									G
320									A
321									G
322									G
323									T
324									C
325			93					T to C	T
326			63					C to T	C
327									G
328									T
329	44, 49, 54, 58, 104		2, 3, 7, 18, 33, 39, 43, 45, 47, 50, 54, 69, 70, 77, 78, 79, 82, 86, 94, 95		12, 34, 69		81	A to G	A
330									G

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation description	WT nucleotide
331							22	A to G	A
332	42	14						C to T	C
333			59					C to T	C
334									G
335							81	T to C	T
336									G
337					11			C to T	C
338		39, 42						A to G	A
339									C
340						21		C to T	C
341	54, 60,	56	8	23	37, 44	7, 68	17	A to C, A to G	A
342		5, 17, 27, 29, 32, 36, 45, 68, 69	5, 15, 20, 63, 74, 76	25, 78, 94		90		T to C, T to A	T
343	8, 23, 72,			4	5			G to C, G to A	G
344									A
345							24	G to A	G
346									C
347									A
348									C
349		21, 40, 73, 74		25		39, 82	8	A to G	A
350						87		A to G	A
351	106	28	19	81		25	64	A to G, A to C	A
352			71					T to C	T
353	31			22, 89				C to T	C
354			62		35			C to T	C
355		5, 49			14, 88	47		T to C	T
356		59	44	2, 46, 57	10, 22, 24, 41, 43, 49, 57, 74, 80, 83, 86, 95		42	A to G	A
357		38						A to G	A
358				71			5	A to G	A
359									C
360		38						C to A	C
361									T
362					85			C to T	C
363									A
364			23	14, 63	34		41	A to G	A
365									A
366				13				G to T	G
367						75	18	A to G	A
368					69			A to G	A
369	1, 103			18				A to C, A to G	A
370	101	24, 41		80				A to T, A to G, A to C	A

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation description	WT nucleotide
371	6, 9, 17, 31, 47, 56, 59, 67, 69, 71, 72, 86, 91, 93	76		8	5, 34		5	A to G, A to T, A to C	A
372		21, 83		22, 91		32, 50, 96	60, 89	C to A, C to T	C
373	22, 44, 49, 54, 61		14, 18, 23, 33, 39, 43, 47, 50, 54, 69, 70, 71, 77, 78, 79, 86, 93, 94, 95	33	34, 69	18	24, 64, 81, 93	C to T, C to A	C
374	59, 72, 86, 93	93	64			82	57	A to T, A to G	A
375	31, 32, 55	38		3, 80	47	18, 74	38	A to G, A to T	A
376	16, 103	73		83	79	64, 87, 91	70, 89	A to C, A to G	A
377	65, 74	22, 50, 59, 96	17, 28,		51, 53, 66, 94	39, 81	11, 15	A to G	A
378							21	G to C	G
379			19, 48, 91		8, 27, 60, 69	68		A to G	A
380	1, 9, 20, 33, 40, 79, 92, 98, 101	21, 31, 40, 73, 74	63	56		82		A to G	A
381		2	3, 5, 9, 16, 22, 37, 52, 58, 61, 65, 67, 68, 72, 74, 76, 82, 85, 92,	69	79	25, 52	3, 50	A to C, A to G, A to T	A
382				95				C to G	C
383	26, 87	65, 66	52, 81	14, 37, 39, 44, 52, 66, 77, 83, 94, 95	2			A to G	A
384							61	C to T	C
385									C
386	43, 69			54, 78	65	92	49	A to G, A to T	A
387	78			68		41		A to G	A
388									C
389		62			33, 43		85	C to T	C
390		18						G to C	G
391				67, 70					T
392	86							C to T	C
393						45		G to A	G
394	10		2, 79		63			C to T	C
395									C
396				27				C to T	C
397	15, 39, 52, 53, 68, 69, 70, 77, 78, 87	58	26, 93				22	A to G, A to C	A
398	79	33	18				94	A to G	A
399	100			72	96			T to C	T
400			33					G to T	G
401	26	47		2			57	A to C, A to G	A
402									T
403							50	T to C	T
404	22							G to C	G

Mutation site	si18-36 experiment 1 (Clone #)	si18-36 experiment 2 (Clone #)	si18-36 experiment 3 (Clone #)	si19-37 experiment 1 (Clone #)	si19-37 experiment 2 (Clone #)	siJFH-1 6367 (Clone #)	Cell-passage control (Clone #)	Mutation description	WT nucleotide
405	61				35		72	A to G, A to T	A
406		89	13	13	52, 81, 82	5		A to G	A
407									C
408				7, 75	44			A to G	A
409	21, 49,		21				61	A to G	A
Deletions									
26		31						Deletion of C	
28	57		77					Deletion of G	
29	1		60		87			Deletion of C	
31			37					Deletion of A	
32			37					Deletion of T	
33	12							Deletion of G	
				69					
118				27, 66, 69	65		14	Deletion of C	
193		73						Deletion of T	
240							13	Deletion of G	
341	106							Deletion of A	
346						86		Deletion of C	
347						86		Deletion of A	
367	15, 101							Deletion of C	
				95					
382		65, 66		37, 44, 83				Deletion of C	
383			2					Deletion of A	
				78					
Insertions									
Between 40 & 41	59					66		Insertion of a A, C	
Between 117 & 118	19, 44, 59	48, 56, 73	20	2, 67, 93	29, 42, 44	90	38, 47, 49	Insertion of a C	
Between 316 & 317			73					Insertion of a G	
Between 352 & 353							66	Insertion of a C	
Between 366 & 367					83	94	26	Insertion of a A	
Between 373 & 374						52	12	Insertion of a A	
Between 377 & 378			73					Insertion of a G	
Between 396 & 397							14	Insertion of a A	
Between 410 & 411							14, 26	Insertion of a A	
Between 412 & 413			73			93		Insertion of a G	